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(54) Title: A METHOD FOR GENERATING HYPERMUTABLE PLANTS

(57) Abstract: Blockade of mismatch repair in a plant can lead to hypermutation and a new genotype and/or phenotype. One approach used to generate hypermutable plants is through the expression of dominant negative alleles of mismatch repair genes in transgenic plants or derived cells. By introducing these genes into cells and transgenic plants, new cell lines and plant varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation. Moreover, methods to inhibit the expression and activity of endogenous plant MMR genes and their encoded products are also useful to generate hypermutable plants.

# A METHOD FOR GENERATING HYPERMUTABLE PLANTS

This application claims the benefit of provisional application Serial No. 60/183,333, filed February 18, 2000.

## TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of mismatch repair genes. In particular it is related to the field of mutagenesis.

## BACKGROUND OF THE INVENTION

Within the past four years, the genetic cause of the Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome II, has been ascertained for the majority of kindreds affected with the disease (1). The molecular basis of HNPCC involves genetic instability resulting from defective mismatch repair (MMR). To date, six genes have been identified in humans that encode proteins which appear to participate in the MMR process, including the *mutS* homologs *GTBP, hMSH2,* and *hMSH3* and the *mutL* homologs *hMLH1*, *hPMS1*, and *hPMS2* (2-7). Germline mutations in four of these genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified in HNPCC kindreds (2-7). Though the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to MMR abnormalities (8,9). In addition to its occurrence in virtually all tumors arising in HNPCC patients, Microsatellite Instability (MI) is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (10).

HNPCC is inherited in an autosomal dominant fashion, so that the normal cells of affected family members contain one mutant allele of the relevant MMR gene (inherited from an affected parent) and one wild-type allele (inherited from the unaffected parent). During the early stages of tumor development, however, the wild-type allele is inactivated through a somatic mutation, leaving the cell with no functional MMR gene and resulting in a profound defect in MMR activity. Because a somatic

mutation in addition to a germ-line mutation is required to generate defective MMR in the tumor cells, this mechanism is generally referred to as one involving two hits, analogous to the biallelic inactivation of tumor suppressor genes that initiate other hereditary cancers. In line with this two-hit mechanism, the non-neoplastic cells of HNPCC patients generally retain near normal levels of MMR activity due to the presence of the wild-type allele (11-12).

While MMR is a conserved process found in bacteria, yeast, and mammalian cells (14-16), its activity has not been confirmed in plants. While sequences homologous to MMR genes have been identified in Arabidopsis thaliana, it is not known if they are functional in plants in the process of MMR (17-18). There is a need in the art for identification of the processes involved in genome stability in plants. There is a continuing need for methods and techniques for generating genetic diversity in agriculturally important crops.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for making a hypermutable cell.

It is another object of the invention to provide a homogeneous composition of cultured, hypermutable, plant cells.

It is still another object of the invention to provide a hypermutable transgenic plant.

It is yet another object of the invention to provide a method for generating a mutation in a gene of interest in a plant cell.

It is still another object of the invention to provide a method for generating a mutation in a gene of interest in a plant.

It is an object of the invention to provide a method for generating a hypermutable plant.

It is another object of the invention to provide a vector for introducing a dominant negative MMR allele into a plant.

It is even another object of the invention to provide an

isolated and purified polynucleotide encoding a plant MutL homolog.

It is another object of the invention to provide an isolated and purified protein which is a plant MutL homolog.

It is an object of the invention to provide a method for determining the presence of a mismatch repair (MMR) defect in a plant or a plant cell.

These and other objects of the invention are provided by one or more of the following embodiments. In one embodiment of the invention a method for making a hypermutable cell is provided. A polynucleotide comprising a dominant negative allele of a mismatch repair gene is introduced into a plant cell, whereby the cell becomes hypermutable.

In another aspect of the invention a homogeneous composition of cultured, hypermutable, plant cells is provided. The plant cells comprise a dominant negative allele of a mismatch repair gene.

Another aspect of the invention is a hypermutable transgenic plant. At least 50% of the cells of the plant comprise a dominant negative allele of a mismatch repair gene.

According to another aspect of the invention a method is provided for generating a mutation in a gene of interest in a plant cell. A hypermutable plant cell comprising the gene of interest and a dominant negative allele of a mismatch repair gene is grown. The cell is tested to determine whether the gene of interest harbors a newly acquired mutation.

Another embodiment of the invention is a method for generating a mutation in a gene of interest in a plant. A plant comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene is grown. The

plant is tested to determine whether the gene of interest harbors a newly acquired mutation.

According to another aspect of the invention a method is provided for generating a hypermutable plant. Endogenous mismatch repair (MMR) activity of a plant is inhibited. The plant becomes hypermutable as a result of the inhibition.

Another aspect of the invention is a vector for introducing a dominant negative MMR allele into a plant. The vector comprises a dominant negative MMR allele under the transcriptional control of a promoter which is functional in a plant.

Still another aspect of the invention provides an isolated and purified polynucleotide encoding Arabidopsis thaliana PMS2 as shown in SEQ ID NO: 14.

Another aspect of the invention provides an isolated and purified polynucleotide encoding Arabidopsis PMS134 as shown in SEQ ID NO: 16.

According to another embodiment of the invention an isolated and purified protein which is Arabidopsis PMS2 is provided. It has the amino acid sequence as shown in SEQ ID NO: 14.

Another embodiment of the invention is an isolated and purified protein which is Arabidopsis PMS134. It has the amino acid sequence as shown in SEQ ID NO: 16.

Still another aspect of the invention provides a method for determining the presence of a mismatch repair (MMR) defect in a plant or a plant cell. At least two microsatellite markers in test cells or a test plant are compared to the at least two microsatellite markers in cells of a normal plant. The test plant or plant cells are identified as having a mismatch repair defect if at least two microsatellite markers are found to be rearranged relative to the cells of the normal plant.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Alignment of the Arabidopsis thaliana and human PMS2 cDNAs.

Fig. 2. Alignment of the Arabidopsis thaliana and human PMS2 proteins.

Fig. 3. Alignment of the Arabidopsis thaliana MLH1 homolog and the human PMS2 proteins.

Fig. 4. Alignment of the Arabidopsis thaliana PMS1 homolog and the human PMS2 proteins.

Fig. 5. Phylogenetic tree of Arabidopsis thaliana MutL homologs and the human PMS2 protein.

Fig. 6. Alignment of the Arabidopsis thaliana PMS134 and the human PMS134 cDNA.

Fig. 7. Alignment of the Arabidopsis thaliana PMS134 and the human PMS134 polypeptides.

Fig. 8. Western blot analysis of bacteria expressing the hPMS134 (Fig. 8A) or the Arabidopsis thaliana PMS134 (Fig. 8B) polypeptides.

Fig. 9. Expression of plant dominant negative MMR genes produces hypermutability in bacteria, demonstrating the functionality of plant MMR proteins.

Fig. 10. Schematic diagram of a plant dominant-negative MMR expression vector.

Fig. 11. Transgenic plants containing the PMS134-KAN vector express the dominant negative hPMS134 gene.

Fig. 12. Microsatellite instability in plants expressing dominant negative MMR hPMS134 gene.

Fig. 13. MMR defective plants produce new phenotypes. Plants with decreased MMR produce offspring with two shoot apical meristems (SAM) in contrast to control plants exhibiting a single SAM.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present inventors that plant cells have functional mismatch repair (MMR) systems which function similarly to mammalian MMR. Moreover, dominant negative alleles can be made and used to generate variability in plants and plant cells, as in mammalian cells. Other means of interfering with normal MMR activity can also be used as described in detail below. Dominant negative alleles of mismatch repair genes, when introduced into cells or plants, increase the rate of spontaneous or induced mutations by reducing the effectiveness of DNA repair and thereby render the cells or whole organism hypermutable. Hypermutable plant cells or plants can be utilized to develop new mutations in a gene of interest.

The process of mismatch repair, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells (9, 14-16). A mismatch repair (MMR) gene is a gene that encodes one of the proteins of a mismatch repair complex. Although not wanting to be bound by any particular theory or mechanism of action, a mismatch repair complex is believed to detect distortions of a DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base, which is complementary to the older DNA strand. In this way, cells eliminate many mutations which occur as a result of mistakes in DNA replication.

For purposes of example, this application discloses use of dominant negative alleles of MMR genes as a method for blocking or inhibiting MMR activity in plants. (Blocking or inhibiting are used synonymously herein, and denote any significant level of inhibition. They do not connote complete inhibition, although the terms include that possibility within their ambit.) However, any molecular method known by those skilled in the art to block MMR gene expression and/or function can be used, including but not limited to gene knockout (19), antisense technology (20), double stranded RNA interference (21), and polypeptide inhibitors (22).

Dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134 (13, U.S. Patent No. 6,146,894) . The mutation causes the product of this gene to prematurely terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele which produces such effect can be used in this invention.

Dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, plants or other organisms as described by Nicolaides et. al. (23) and Hori et. al. (24). Alternatively such alleles can be made from wild-type alleles, typically by inserting a premature stop codon or other mutation which leads to a protein product which is able to complex with other members of the MMR complex but which is not functional. Such alleles can be identified by screening cells for defective mismatch repair activity. The cells may be mutagenized or not. Cells from plants exposed to chemical mutagens or radiation, *e.g.*, can be screened for defective mismatch repair. Genomic

DNA, a plasmid containing cDNA, or mRNA from any cell encoding a mismatch repair protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other mismatch repair genes (13, U.S. Patent No. 6,146,894). Other truncated alleles of *PMS2* or other MMR genes can be made. Such alleles are expected to behave similarly to *hPMS2-134*. An of various forms of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or plants can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if the allele is dominant negative.

A cell or a plant into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the mutation rate (spontaneous or induced) of such cells or plants is elevated compared to cells or plants without such alleles. The degree of elevation of the mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or plant.

According to one aspect of the invention, a polynucleotide encoding a dominant negative form of a mismatch repair protein is introduced into a cell or a transgenic plant. The gene can be any dominant negative allele encoding a protein which is part of a mismatch repair complex, for example, *mutS or mutL* homologs of the bacterial, yeast, fungal, insect, plant, or mammalian genes. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide. The polynucleotide can be introduced into the cell by transfection.

Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out in a living plant,

*e.g.*, using a binary vector for gene transmission, or it can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any type of plant cell.

In general, transfection can be carried out using a suspension of cells, or a single cell, but other methods can also be used as long as a sufficient fraction of the treated cells incorporates the polynucleotide to allow transfected cells to be readily isolated. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known in the art of plant cell science. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, Agrobacterium-mediated gene transfer, cell fusion, the use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the mismatch repair gene, the cell can, *e.g.*, be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results. Alternatively, a dominant negative MMR protein can be directly introduced by microinjection into a cell in order to inhibit MMR activity of the cell.

Root explants are incubated in 0.5 ug/ml of 2-4-dochlorophenoxy-acetic acid (2-4D) plus N6-Benzyl-Adenine in growth medium. After 4 weeks, suspension cells are isolated and digested with hemicellulase for protoplast preparation and transfection. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a multicellular plant in the form of a primary cell culture or an immortalized cell line, or may be derived from suspensions of single-celled plants.

A polynucleotide encoding a dominant negative form of a mismatch repair protein can be introduced into the genome of a plant to form a transgenic plant. The plant can be any species for which suitable

techniques are available to produce transgenic plants. For example, transgenic plants can be prepared from domestic agricultural crops, *e.g.* corn, wheat, soybean, rice, sorghum, barley, etc.; from plants used for the production of recombinant proteins, *e.g.*, tobacco leaf; or experimental plants for research or product testing, *e.g.*, *Arabidopsis*, pea, *etc.* The introduced polynucleotide may encode a protein native to the species or native to another species, whether plant, animal, bacterial, or fungal, for example.

Any method for making transgenic plants known in the art can be used. According to one process of producing a transgenic plant, the polynucleotide is transfected into the plant seedling The seed is germinated and develops into a mature plant in which the polynucleotide is incorporated and expressed. An alternative method for producing transgenic plants involves introducing the polynucleotide into the growing or mature plant by injection, electroporation, Agrobacterium-mediated transfer or transfection. With this method, if the polynucleotide is not incorporated into germline cells, the gene will not be passed on to the progeny. Therefore, a transgenic plant produced by this method will be useful to produce products from that individual plant.

To identify whether a gene was inserted into the germline, seedlings derived from such plants can be screened for the transgene. Genetic modification of a growing or mature plant is useful for evaluating the expression of hypermutable constructs and for evaluating effects on altering endogenous mismatch repair. Once transgenic plants are produced, they can be grown to produce and maintain a crop of transgenic plants.

Once a transfected cell line or a crop of transgenic plants has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic plant or introduced into the cell line or transgenic plant. An advantage of using MMR-defective cells or plants to induce mutations is that the cell or plant need not be exposed to mutagenic

chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers.

Mutations can be detected by analyzing the genotype of the cells or plants, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by testing a phenotype caused by the gene. A mutant phenotype can be detected, *e.g.*, by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or plant associated with the function of the gene of interest or its protein product. Finally, one can screen for macroscopic phenotypes such as but not limited to color, height, or the ability to grow in drought, high-salt, cold, hot, acidic, basic, pest-infested, or high ethylene environments.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples that will be provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: Isolation of Plant Mismatch Repair Genes.

The ability to increase the hypermutability of host genomes has many commercial and medical applications. The generation of hypermutable plants such as those used in agriculture for livestock feed and human consumption are just one example of many types of applications that can be generated by creating hypermutable organisms. For instance, the creation of crops that are less susceptible to pests or soil pH would greatly increase yield of certain agricultural crops. In addition to greater production of goods, improved crops could increase the ability to grow many generations of crops on the same fields (25-27). Moreover, the

ability to affect certain growth traits such as natural pest-resistance, drought-resistance, frost-resistance, increased production, or altered stalk size has many benefits for the production of agricultural products. Recently, it has been demonstrated that genes affecting the biologic activity of the plant growth hormone gibberellin results in crops with shorter stalk length that produce similar amounts of grain yields, however, the fact that the stalks are shorter makes these plants less susceptible to high winds and crop damage (28). The use of hypermutable crops could allow for the selection of shorter plants across many species such as corn, rice, etc, without having to first identify a gene to alter its activity. Another application of hypermutable agricultural products is the generation of crops with enhanced levels of vitamins and nutrients. One can select for enhanced vitamin production in seedlings of MMR defective plants. Recently, it has been demonstrated that altering a gene(s) within a vitamin biosynthetic pathway can result in the production of elevated levels of vitamin E (27,29).

Applications of hypermutable plants include use as crops for agricultural production, increased medicinal entities within plant extracts, chemicals and resins for industrial use, and their use as detoxifying organisms for environmental applications as described (25,26,29).

MutS and mutL homologs can be isolated from plant species libraries using degenerate RT-PCR, and standard Southern hybridization techniques as previously described (3,23,30). These may serve as reagents for producing MMR defective plant hosts. This process employs methods known by those skilled in the art of gene cloning.

One such approach is the use of degenerate PCR to clone MutS homologs following the methods used by Leach et. al. to clone the human MSH2 (3). Additional degenerate oligonucleotides can be generated and used against conserved domains of bacterial, yeast, and human MutS homologs. Various plant species cDNAs (obtainable from various commercial sources) can be amplified for MutS gene homologs by

polymerase chain reaction (PCR). Products are cloned into T-tailed vectors (In Vitrogen) and analyzed by restriction endonuclease digestion. Clones with expected DNA fragment inserts are sequenced using M13 forward and reverse primers located on the vector backbone flanking the cloning site. Fragments containing MMR gene homologs are then used as probes to screen commercially available cDNA libraries from the appropriate species. cDNA contigs are generated to create a cDNA containing the sequence information for the full length MMR gene and its encoded polypeptide. One such example of cloning a plant MMR gene is provided below.

In order to clone mutL homologs, degenerate primers were synthesized to the conserved domains of the mutL gene family by aligning *E. coli*, yeast, mouse, and human *mutL* genes. These primers are directed to the polynucleotide sequences centered at nt 150 to 350 of the published human PMS2 cDNA (SEQ ID NO: 3). Degenerate PCR was carried out using RNA from Arabidopsis thaliana (AT) that was isolated using the RNeasy kit following the manufacturer's protocol (Qiagen). RNAs were reverse transcribed (RT) using SuperscriptII (Life Technologies) following the manufacturer's protocol. After RT, cDNAs were PCR amplified using degenerate primers in buffers described by Nicolaides et. al. 1995 (23,30), and reactions were carried out at 95°C for 30 sec for 1 cycle followed by 94°C for 30 sec, 45°C for 60 sec, and 72°C for 60 sec for 20 cycles. PCR reactions were then diluted 1:10 in water and reamplified using the same primers and buffers. The secondary PCR reactions were carried out at 95°C for 30 sec for 1 cycle followed by 94°C for 30 sec, 52°C for 90 sec, and 72°C for 90 sec for 35 cycles. Reactions were analyzed by agarose gel electrophoresis. Products of the expected molecular weight were excised and cloned into T-tailed vectors (InVitrogen). Recombinant clones were sequenced and blasted against the public databases. The homolog was found to have homology to the mutL family of genes. Blast search analysis of GenBank found this gene to be part of a "putative" mismatch repair gene identified from the Arabidopsis genome project that has never been

reported to be transcribed or capabl of producing a message. In order to clone the full length, an Arabidopsis cDNA library was screened by PCR as well as cDNA from AT plants using 5' primers corresponding to the initiation codon (SEQ ID NO: 1: 5'-atg caa gga gat tct tc-3') and the termination codon (SEQ ID NO: 2: 5'-tca tgc caa tga gat ggt tgc-3') using buffers and conditions listed above. Amplifications were carried out at 95°C for 30 sec for 1 cycle followed by 94°C for 30 sec, 58°C for 2 min, and 72°C for 3 min for 35 cycles. Products were analyzed by gel electrophoresis. Products of the expected molecular weights were subcloned into T-tail vectors and sequenced using primers from the cloning vector or using internal primers. Figure 1 shows the alignment of one Arabidopsis homolog, referred to as *ATPMS2* (SEQ ID NO: 4), to the human *PMS2* cDNA (SEQ ID NO:3) (Fig. 1) and the hPMS2 protein (Fig. 2; SEQ ID NO:13). This gene was found to be homologous (48% identity) to the human *PMS2* (SEQ ID NO:3) cDNA and its encoded polypeptide (31% identity) (Figure 2). Other homologs to the *ATPMS2* were also identified from blast searching sequence databases. One mutL homolog is closely related to the MLH1 mammalian homolog and is referred to as ATMLH1 (shown in Fig. 3) and another is closely related to the mammalian PMS1 polypeptide referred to as ATPMS1 (shown in Fig. 4). A phylogenetic tree is shown in Fig. 5 showing the homology of the mutL homologs to the human *PMS2* gene.

Degenerate primers can be used for isolating MMR genes from other plant species in a similar fashion.

EXAMPLE 2: Generation Of Dominant Negative Alleles Of Plant Mismatch Repair Genes

To demonstrate that putative plant MMR proteins are truly involved in MMR biochemical process, cDNAs are cloned into constitutive (31,32) or inducible (33) bacterial expression vectors for functional studies.

Various deletion mutants are generated to produce dominant negative MMR genes. Dominant negative alleles that are identified in the bacterial system are then useful for plant studies. Dominant negative MMR genes are prepared by over-expression of full-length MMR genes or by deletion analysis using standard protocols used by those skilled in the art of molecular biology. One such dominant MMR gene mutant was created by generating a construct with similar domains to that of the human dominant negative PMS2 gene (referred to as PMS134) (13, U.S. Patent No. 6,146,894). To generate this vector, the ATPMS2 (SEQ ID NO: 4) and hPMS2 cDNA (SEQ ID NO: 3) sequences were aligned and the conserved domain was isolated. Figure 6 shows a sequence alignment between the human and AT PMS134 cDNAs where a 52% identity is found between the two sequences. At the protein level these domains have a 51% identity (Figure 7). Dominant negative hPMS134 and ATPMS134 genes were made by PCR and subcloned into bacterial expression vectors. The ATPMS134 was generated by PCR from the cloned cDNA using a sense primer (SEQ ID NO:1) corresponding to the N-terminus and an antisense primer (SEQ ID NO:5) 5'gtcgacttatcacttgtcatcgtcgtccttgtagtcgagcgtagc-aactggctc-3' centered at nt 434 of the ATPMS2 cDNA (SEQ ID NO:4). This primer also contains a flag epitope that will allow protein detection followed by two termination codons. PCR products of the expected molecular weight were gel purified and cloned into T-tail vectors. Recombinant clones were sequenced to ensure authentic sequences. Inserts were then cloned into the inducible pTAC expression vector, which also contains the Ampicillin resistance gene as a selectable marker. The human PMS134 allele was also cloned into the pTAC expression vector as a positive control. Electrocompetent DH5alpha and DH10b bacterial cells (Life Technologies) were electroporated with empty vector, and the loaded vectors pTACATPMS134 and pTAChPMS134, using an electroporator (BioRAd) following the manufacturer's protocol. Bacterial cultures were then plated on to LB agar plates containing 100μg/ml ampicillin and grown

at 37°C for 14 hours. Ten recombinant clones were then isolated and grown in 5 mls of LB broth containing 50 μg/ml ampicillin plus 50μM IPTG for 18 hr at 37°C. One hundred microliters were then collected, spun down, and directly lysed in 2X SDS buffer for western blot analysis. For western analysis, equal number of cells were lysed directly in 2X SDS buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins are separated by electrophoresis on 4-12% NuPAGE gels (Novex). Gels are electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters are probed with a polyclonal antibody generated against MMR polypeptide sequence or a fused tag (e.g. FLAG, HIS, etc.) and a horseradish peroxidase conjugated secondary antibody, using chemiluminescence for detection (Pierce). Figure 8 shows a western blot of a clone that expresses the human PMS134 protein (Figure 8A, lane 2) using a human PMS2-specific antibody (directed to residues 2-20) of the hPMS134 sequence (see Fig. 1, and SEQ ID NO:6) or the Arabidopsis PMS134 protein (Figure 8B, lane 2) using an anti-FLAG antibody directed to the fusion residues at the C-terminus of the protein. Cells expressing empty vector had no detectable expression.

Bacterial clones expressing the h*PMS134, ATPMS134* or the empty vector were grown in liquid culture for 24 hr at 37°C in the presence of 50 μg/ml ampicillin plus 50μM IPTG. The next day, cultures were diluted 1:10 in medium containing 50μM IPTG plus ampicillin or ampicillin plus 25 μg/ml kanamycin (AK) and cultures were grown for 18 hr at 37°C. The following day, a 0.1 μl aliquot (2 μl diluted in 1000 μl of LB medium and used 50 μl for plating) of cells grown in Amp medium were plated on LB-agar plates containing 40 μg/ml of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) plus 100μg/ml ampicillin (AMP), while a 1 μl aliquot (1 μl diluted in 100 μl of LB medium and used 100 μl for plating) of cells

grown in AK medium were plated on LB-agar plates containing X-gal and 50μg/ml kanamycin (KAN). Plates were incubated for 18 hours at 37°C. The results from these studies show that cells expressing the hPMS134 or the ATPMS134 polypepetides displayed increased mutation rates in the genome of the DH10B bacterial strain which resulted in the production of KAN resistant clones (Figure 9). Following the mutagenesis protocol described above, bacterial cells expressing the plant ATPMS134 were found to have an increase in the number of KAN resistant cells (12 clones) in contrast to cells expressing the empty vector, which yielded no KAN resistant clone. These data demonstrate that dominant negative *ATPMS134* MMR genes are useful for creating hypermutable organisms that can generate phenotypically diverse offspring when put under selective conditions. Moreover, these data demonstrate that plants also use the conserved MMR process for genomic stability.

Dominant negative plant MMR gene mutants are also analyzed using mammalian cell systems. In this case, plant MMR gene cDNAs are cloned into eukaryotic expression vectors as described (13,34) and cells expressing dominant negative mutants are analyzed by measuring stability of endogenous microsatellite markers and biochemical activity of cell extracts from lines expressing dominant negative MMR gene alleles. Such methods are known by those skilled in the art and previously described (13).

EXAMPLE 3: Inhibition Of Plant MMR Activity By Dominant Negative MMR Alleles Produces Genetic Hypermutability And Microsatellite Instability.

Dominant negative alleles of human and AT MMR genes identified using bacterial and or mammalian systems can be used for plants. To test the hypothesis that dominant negative MMR gene

alleles produce global hypermutability in plants, the hPMS134 and ATPMS134 cDNAs were expressed in plants. These alleles have been found to work across species where the introduction of these genes into MMR proficient bacterial or mammalian cells renders the cells MMR deficient. Assays to carry out these studies are described below.

**Engineering plant expression vectors to express the PMS134 dominant negative alleles.**

A BamH I fragment containing the hPMS134 cDNA was obtained from the pSG5PMS134 plasmid (ref 13) and cloned into the corresponding sites of the pEF1/SP1-V5 vector (InVitrogen). The resulting vector (pEF-PMS134-sense) was then digested with Pme I to release a blunted DNA fragment containing the PMS134 cDNA. This fragment was then subcloned into the blunt Sma I and EcoICR I sites of the pGPTV-KAN binary plant expression vector (American Type Culture Collection). One clone, named pCMV-hPMS134-Kan (see figure 10), was sequenced to confirm that the vector contained authentic promoter and gene insert sequences. A schematic diagram of the pCMV-hPMS134-Kan vector is shown in Figure 10.

**Generation of hPMS134-Expressing Arabidopsis thaliana transgenic plants.**

*Agrobacterium tumefaciens* cells (agrobacteria) are used to shuttle genes into plants. To generate PMS134-expressing Arabidopsis thaliana (A. thaliana) plants, *Agrobacterium tumefaciens* cells (strain GV3101) were electroporated with pCMV-hPMS134-Kan or the pBI-121 (BRL) control binary vector. The pBI-121 control contains the CaMV promoter driving the expression of the β-glucuronidase cDNA (GUS) and serves as a control. Both vectors carry the neomycin phosphotransferase (NPTII) gene that allows selection of agrobacteria and plants that contain the expression vector. One-month old A. thaliana (ecotype Columbia) plants were infected

by immersion in a solution containing 5% sucrose, 0.05% silwet, and binary vector-transformed agrobacteria cells for 10 seconds. These plants were then grown at 25°C under a 16 hour day and 8 hour dark photoperiod. After 4 weeks, seeds (referred to as T1) were harvested and dried for 5 days at 4°C. Thirty thousands seeds from ten CMV-hPMS134-Kan-transformed plants and five thousand seeds from two pBI-121-transformed plants were sown in solid Murashige and Skoog (MS) media plates in the presence of 50 μg/ml of kanamycin (KAN). Three hundred plants were found to be KAN resistant and represented PMS134 expressing plants. These plants along with 300 control plants were grown in MS media for two weeks and then transferred to soil. Plants were grown for an additional four weeks under standard conditions at which time T2 seeds were harvested.

To confirm the integration and stability of the PMS134 gene in the plant genome, gene segregation and PCR analyses were conducted. Commonly, three out of four T1 plants transformed by agrobacteria technology are expected to carry the vector inserted within a single locus and are therefore considered heterozygous for the integrated gene. Approximately 75% of the seeds (T2) generated from most of the T1 plants were found KAN–resistant and this in accordance with the expected 1:2:1 ratio of null (no hPMS134 containing plants), heterozygous, and homozygous plants, respectively, in self-pollinating conditions. To confirm that these plants contained the hPMS134 expression vector, genomic DNA was isolated from leaves of T1 plants using the DNAzol-mediated technique following the manufacturer's suggestion (BRL-Life Technologies). One nanogram of genomic DNA was analyzed by polymerase chain reaction (PCR) to confirm the presence of the hPMS134 gene. PCR was carried out for 25 cycles with the following parameters: 95°C for 30 seconds; 55°C for 1 minute; and 72°C for 2 minutes using hPMS134-specific sense (SEQ ID NO: 7: 5'-tct aga cat gga gcg agc tga gag ctc-3') and antisense (SEQ ID NO: 8: 5'-tct aga agt tcc aac ctt cgc cga tgc-3') primers. Positive reactions were observed in DNA from pCMV-

hPMS134-Kan-transformed plants and not from pBI-121-transformed plants, thus confirming the integration of this vector.

In order to assess the expression of hPMS134 in T1 plants, leaf tissue was collected from T1 plants, homogenized in liquid nitrogen using glass pestles, and suspended in RLT lysing buffer (Qiagen, RNeasy plant RNA extraction kit). Five micrograms of total RNA was purified according to the manufacturer's suggested protocol and then loaded onto a 1.2% agarose gel (1x MOPS buffer, 3% formaldehyde), size-fractionated by electrophoresis, and transferred onto N-Hybond+ membrane (Amersham). Each membrane was incubated at 55$^0$C in 10 ml of hybridization solution (North2South labeling kit, Pierce) containing 100 ng of PMS134, tubulin, or KAN cDNA probes, which were generated by PCR amplification, according to the manufacturer's directions. Membranes were washed three times in 2x SSC, 0.1% SDS at 55$^0$C, and three times in 2x SSC at ambient temperature. Detection was carried out using enhanced chemiluminescence (ECL). Expression was also carried out by reverse trascriptase PCR as described above using polyA isolated mRNA that was isolated over a oligo dT column (Qiagen). A representative example of these studies are shown in figure 11. Here hPMS134 expression was detected in three out of ten analyzed pCMV-hPMS134-Kan transgenic lines, while no signal was found in the ten pBI-121 transformed plants analyzed. Immunoblot using whole lysates is used to confirm protein expression. Collectively these studies demonstrate the generation of hPMS134 expressing transgenic A. thaliana plants.

**Molecular Characterization of PMS134-Expressing Plants.**

MMR is a process that is involved in correcting point mutations and "slippage" mutations within repetitive mono-, di-, and tri-nucleotide (microsatellite) repeats that occur throughout the genome of an organism after cellular replication. This process is very similar to a computer spell

check function. The inactivation of MMR has been sh wn to result in global genomic hypermutation whereby cells with defective MMR experience over a one thousand-fold increase in point mutations and microsatellite instability (MI) (mutations within repetitive sequences) throughout their genomes per division. (35). MMR deficiency is the only known process capable of producing MI and has been used as a marker to detect cells with MMR dysfunction (36). Microsatellites serve as molecular tools to measure the inactivation of MMR that occurs by the defective MMR due to but not limited to expression of dominant negative MMR genes, double stranded RNA interference vectors, or inhibition by antisense nucleotides, or by gene knockout. In *A. thaliana*, a series of poly-A (A)n, (CA)n and (GA)n sequences were identified from genome searches using EMBL and GenBank databases. To demonstrate that hPMS134 expression could produce MI in *A. thaliana*, we analyzed microsatellites in T1 plants by PCR analyses. Initially we monitored three microsatellites, ATHACS, Nga280, and Nga128 with published primers that have been previously used to map the Arabidopsis genome (37). Briefly, DNA was extracted from A. thaliana leaves as described above. 10 ngs of plant genomic DNA was used as template for PCR amplification using the following amplification conditions: 94°C for 15 sec, 55°C for 15 sec and 72°C for 30 seconds. PCR products were analyzed on 5% Metaphor agarose (BioWhittaker Molecular Applications) and ethidium bromide staining. In one transgenic pCMV-PMS134-Kan line, we detected a double product, likely representing a new allele of the polymorphic nga280 locus (Figure 12). These data demonstrate the ability to produce MMR deficiency and MI in plants expressing the hPMS134 dominant negative allele and provide a molecular tool for screening MMR-defective plants.

Biochemical assays for mismatch repair. MMR activity in nuclear extracts is performed as described, using 24 fmol of substrate as described (13). Complementation assays are done by adding ~ 100 ng of purified MutL or MutS components to 100 μg of nuclear extract, adjusting the final

KCl concentration to 100 mM. The substrates used in these experiments will contain a strand break 181 nucleotides 5' or 125 nucleotides 3' to the mismatch.

EXAMPLE 4: Inactivation Of MMR Leads To Plants With New Phenotypes.

We demonstrated the ability of the defective MMR to produce molecular changes within plants. The objective of this section is to demonstrate the ability to generate MMR defective plants with macroscopic output traits. One way to measure for plants with new phenotypes is to grow plants under toxic conditions, such as but not limited to high levels of toxic ions, pest-infection, drought conditions, or extreme temperatures to identify a minority of plants with new output traits, *i.e.*, resistance. Another way to score for plants with new phenotypes is through physical differences of MMR defective plants grown in standard conditions. An example of MMR-defective plants with new phenotypes include the generation of plants with double shoot apical meristems (Figure 13) as well as plants with altered chlorophyll production rendering plants albino (data not shown). In Figure 13, we show a typical wild type plant (left, labeled normal) and a plant produced from the MMR defective group (right, labeled MMR deficient). The double-meristem trait was not observed in greater than 500 normal plants. The double-meristem trait does not appear to be due to transgene integration since segregation analysis reveals the ability to generate double-meristem plants in the absence of transgene positive plants while MMR proficient control plants with other transgene vectors (pBI-121) did not produce this phenotype (data not shown). These data suggest that defective MMR produced a mutation or mutations within the plant genome that altered the normal biochemical function of the host to produce a new output trait.

These data demonstrate the ability to create plant subtypes with new

genetic and phenotypic traits by blocking the endogenous MMR process of the plant cell or whole organism.

EXAMPLE 5: Inhibition of Plant MMR Activity Using Molecular Approaches.

This application teaches of the use of inhibiting MMR activity in a plant to produce genetically altered offspring with new phenotypes.

The inhibition of MMR activity in a host organism can be achieved by introducing a dominant negative allele as shown in Figure 11 and 12. Other ways to suppress MMR activity of a host is by: knocking out alleles of a MMR protein through homologous recombination (38); blocking MMR protein dimerization with other subunits (which is required for activity) by the introduction of polypeptides into the host via transfection methods; knocking out the expression of a MMR gene using anti-sense oligonucleotides (20), and/or the use of double stranded RNA interference genes (21).

MMR gene knockouts.

Data shown in EXAMPLE 1 demonstrate that plants contain MMR gene homologs that can be genetically engineered to produce altered biochemical activities. Data presented in EXAMPLES 3 and 4 demonstrate that defective MMR in plants can produce hypermutable parental plants and offspring. Together, these data demonstrate that inhibiting endogenous MMR genes by targeting vectors of the particular MMR gene can lead to hypermutability of a plant host that generate offspring with altered genetic loci and/or new phenotypes as described in EXAMPLES 3, 4, and 5. Hypermutable seedlings can also be produced with "knocked out" MMR genes using methods known by those skilled in the art. These can be used to produce genetically diverse offspring for commercial and medical

applications (38). C lls will be confirmed to have lost the expression of th MMR gene using standard northern techniques and determined to be MMR defective using microsatellite analysis as described in EXAMPLE 3.

Blocking polypeptides.

MMR subunits (MutS and MutL proteins) interact to form active MMR complexes. Peptides are able to specifically inhibit the binding of two proteins by competitive inhibition. Isolation of plant MMR genes allows for the elucidation of primary amino acid structures as described in EXAMPLE 1. Peptides containing some but not all of the domains can be synthesized from domains of the particular MMR gene and introduced into host plants using methods known by those skilled in the art (22). Like truncated PMS134, such peptides will compete with functional full length proteins for binding and form enzymatically inactive MMR complexes. The data indicate that the domains which are C-terminal to the 134 position in human PMS2 are dispensible for binding and necessary for enzymatic activity. As shown herein, a similar domain structure is also found in plant PMS2. Seedlings exhibiting hypermutability will be useful to produce genetically diverse offspring for commercial and medical applications.

RNA blockade and Double Stranded Interference.

MMR subunits (MutS and MutL proteins) interact to form active MMR complexes. Peptides are able to specifically inhibit the binding of two proteins by competitive inhibition. Antisense oligonucleotides are synthesized against the cDNA sequence of plant MMR homologs identified in EXAMPLE 1 (20). Antisense molecules are then introduced into host plants using methods described in EXAMPLE 2 or through the bathing of seedlings or plantlets. Seedlings exhibiting hypermutability will be useful to produce genetically diverse offspring for commercial and medical applications.

Double stranded interference vectors are also useful for blocking expression/function of a plant MMR gene. The plant gene is expressed in both sense and antisense orientations from a transfection vector and the endogenous gene expression is suppressed by endogenous silencing processes (21).

Discussion

Plants contain MMR genes that code for MMR functional proteins. Expression of dominant negative plant MMR proteins results in an increase in microsatellite instability and hypermutability in plants. This activity is due to the inhibition of MMR biochemical activity in these hosts. The data provided within this application demonstrates the blockade of MMR in a plant to produce genetic changes that lead to the production of offspring or cells with new output traits. This method is applicable to generate crop plants with new output traits as well as plant cells exhibiting new biochemicals for commercial use.

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WE CLAIM:

1. A method for making a hypermutable cell, comprising the step of:

introducing into a plant cell a polynucleotide comprising a dominant negative allele of a mismatch repair gene, whereby the cell becomes hypermutable.

2. The method of claim 1 wherein the polynucleotide is introduced by transfection of a suspension of plant cells *in vitro*.

3. The method of claim 1 wherein the mismatch repair gene is a plant *MutS* homolog.

4. The method of claim 1 wherein the mismatch repair gene is a plant *MutL* homolog.

5. The method of claim 1 wherein the mismatch repair gene is a mammalian *PMS2*.

6. The method of claim 1 wherein the mismatch repair gene is a mammalian *MLH1*.

7. The method of claim 1 wherein the mismatch repair gene is a mammalian *PMS1*.

8. The method of claim 1 wherein the mismatch repair gene is a mammalian *MSH2*.

9. The method of claim 1 wherein the mismatch repair gene is an eukaryotic *mutS*.

10. The method of claim 1 wherein the mismatch repair gene is an eukaryotic *mutL*.

11. The method of claim 1 wherein the mismatch repair gene is a prokaryotic *mutS*.

12. The method of claim 1 wherein the mismatch repair gene is a prokaryotic *mutL*.

13. The method of claim 3 wherein the allele comprises a truncation mutation.

14. The method of claim 4 where the allele comprises a truncation mutation.

15. The method of claim 5 where the allele comprises a truncation mutation.

16. The method of claim 15 wherein the allele comprises a truncation mutation at codon 134.

17. The method of claim 16 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type human *PMS2*.

18. The method of claim 1 wherein the polynucleotide is introduced into a plant cell in a plant to form a transgenic plant.

19. The method of claim 18 further comprising: growing the transgenic plant to form a mature transgenic plant.

20. The method of claim 19 wherein the mismatch repair gene is *PMS2*.

21. The method of claim 19 wherein the mismatch repair gene is a mammalian *PMS2*.

22. The method of claim 19 wherein the mismatch repair gene is a mammalian *MLH1*.

23. The method of claim 19 wherein the mismatch repair gene is a mammalian *PMS1*.

24. The method of claim 19 wherein the mismatch repair gene is a mammalian *MSH2*.

25. The method of claim 19 wherein the mismatch repair gene is a plant *MutS* homolog.

26. The method of claim 19 wherein the mismatch repair gene is a plant *MutL* homolog.

27. The method of claim 19 wherein the mismatch repair gene is an eukaryotic *MutS* homolog.

28. The method of claim 19 wherein the mismatch repair gene is an eukaryotic *MutL* homolog.

29. The method of claim 19 wherein the mismatch repair gene is a prokaryotic *MutS* homolog.

30. The method of claim 19 wherein the mismatch repair gene is a prokaryotic *MutL* homolog.

31. The method of claim 20 wherein the allele comprises a truncation mutation.

32. The method of claim 20 wherein the allele comprises a truncation mutation at codon 134.

33. The method of claim 20 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type h*PMS2*.

34. A homogeneous composition of cultured, hypermutable, plant cells which comprise a dominant negative allele of a mismatch repair gene.

35. The homogeneous composition of claim 34 wherein the mismatch repair gene is *PMS2*.

36. The homogeneous composition of claim 34 wherein the mismatch repair gene is mammalian *PMS2*.

37. The homogeneous composition of claim 34 wherein the mismatch repair gene is mammalian *MLH1*.

38. The homogeneous composition of claim 34 wherein the mismatch repair gene is mammalian *PMS1*.

39. The homogeneous composition of claim 34 wherein the mismatch repair gene is mammalian *MSH2*.

40. The homogeneous composition of claim 34 wherein the mismatch repair gene is a plant MutS homolog.

41. The homogeneous composition of claim 34 wherein the mismatch repair gene is a plant MutL homolog.

42. The homogeneous composition of claim 34 wherein the mismatch repair gene is an eukaryotic MutS homolog.

43. The homogeneous composition of claim 34 wherein the mismatch repair gene is an eukaryotic MutL homolog.

44. The homogeneous composition of claim 34 wherein the mismatch repair gene is a prokaryotic MutS homolog.

45. The homogeneous composition of claim 34 wherein the mismatch repair gene is a prokaryotic MutL homolog.

46. The homogeneous composition of claim 34 wherein the cells express a protein consisting of the first 133 amino acids of hPMS2.

47. A hypermutable transgenic plant wherein at least 50% of the cells of the plant comprise a dominant negative allele of a mismatch repair gene.

48. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a plant *MutS*.

49. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a plant *MutL*.

50. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a mammalian *MutS* homolog.

51. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a mammalian *MutL* homolog.

52. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is an eukaryotic *MutS* homolog.

53. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is an eukaryotic *MutL* homolog.

54. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a prokaryotic *MutS* homolog.

55. The hypermutable transgenic plant of claim 47 wherein the mismatch repair gene is a prokaryotic *MutL* homolog.

56. The hypermutable transgenic plant of claim 47 comprising a protein which consists of the first 133 amino acids of human *PMS2*.

57. A method for generating a mutation in a gene of interest in a plant cell, comprising the steps of:

growing a hypermutable plant cell comprising the gene of interest and a dominant negative allele of a mismatch repair gene;

testing the cell to determine whether the gene of interest harbors a mutation.

58. The method of claim 57 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

59. The method of claim 57 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

60. The method of claim 57 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

61. The method of claim 57 wherein the step of testing comprises analyzing a phenotype caused by the gene of interest.

62. The method of claim 57 wherein the plant cell is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a plant cell, whereby the cell becomes hypermutable.

63. The method of claim 62 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

64. The method of claim 62 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

65. The method of claim 62 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

66. The method of claim 62 wherein the step of testing comprises analyzing a phenotype caused by the gene of interest.

67. A method for generating a mutation in a gene of interest in a plant, comprising the steps of:

growing a plant comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene;

testing the plant to determine whether the gene of interest harbors a mutation.

68. The method of claim 67 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

69. The method of claim 67 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

70. The method of claim 67 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

71. The method of claim 67 wherein the step of testing comprises analyzing a phenotype caused by the gene of interest.

72. The method of claim 67 wherein the plant is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a plant, whereby the plant becomes hypermutable.

73. The method of claim 72 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

74. The method of claim 72 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

75. The method of claim 72 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

76. The method of claim 72 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

77. A hypermutable transgenic plant made by the method of claim 67.

78. The hypermutable transgenic plant of claim 77 wherein the mismatch repair gene is *PMS2*.

79. The hypermutable transgenic plant of claim 77 wherein the mismatch repair gene is human *PMS2*.

80. The hypermutable transgenic plant of claim 77 wherein the mismatch repair gene is human *MLH1*.

81. The hypermutable transgenic plant of claim 77 wherein the mismatch repair gene is human *PMS1*.

82. The hypermutable transgenic plant of claim 77 wherein the mismatch repair gene is human *MSH2*.

83. The hypermutable transgenic plant of claim 77 wherein the allele comprises a truncation mutation.

84. The hypermutable transgenic plant of claim 77 wherein the allele comprises a truncation mutation at codon 134.

85. The hypermutable transgenic plant of claim 83 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2*.

86. A method for generating a hypermutable plant, comprising the steps of:

inhibiting endogenous mismatch repair (MMR) activity of a plant, whereby the plant becomes hypermutable.

87. The method of claim 86 wherein an endogenous plant MutS homolog is inhibited by mutagenizing an allele encoding the MutS homolog by introducing a mutation into said allele by homologous recombination.

88. The method of claim 86 wherein an endogenous plant MutL homolog is inhibited by mutagenizing an allele encoding the MutL homolog by introducing a mutation into said allele by homologous recombination.

89. The method of claim 86 wherein an endogenous plant MutL homolog is inhibited by introduction of a dominant negative allele of a plant MutL gene.

90. The method of claim 86 wherein an endogenous plant MutS homolog is inhibited by introduction of a dominant negative allele of a plant MutS gene.

91. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing into said plant inhibitory peptides derived from plant MutS proteins.

92. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing into said plant inhibitory peptides derived from plant MutL proteins.

93. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing into said plant antisense *MutS* oligodeoxynucleotides.

94. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing into said plant antisense *MutL* oligodeoxynucleotides.

95. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing a polynucleotide encoding a MutS polypeptide from a lower organism into said plant and overexpressing in said plant the MutS polypeptide from the lower organism.

96. The method of claim 86 wherein wherein endogenous MMR activity is inhibited by introducing a polynucleotide encoding a MutL polypeptide from a lower organism into said plant and overexpressing in said plant the MutL polypeptide from the lower organism.

97. The method of claim 95 wherein the lower organism is a bacterium.

98. The method of claim 95 wherein the lower organism is a yeast.

99. The method of claim 95 wherein the lower organism is a unicellular organism.

100. The method of claim 96 wherein the lower organism is a bacterium.

101. The method of claim 96 wherein the lower organism is a yeast.

102. The method of claim 96 wherein the lower organism is a unicellular organism.

103. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing a polynucleotide encoding a MutL polypeptide from a rodent into said plant and overexpressing in said plant the MutL polypeptide from the rodent.

104. The method of claim 86 wherein endogenous MMR activity is inhibited by introducing a polynucleotide encoding a MutS polypeptide from a rodent into said plant and overexpressing in said plant the MutS polypeptide from the rodent.

105. The method of claim 86 wherein endogenous MMR activity is inhibited by double stranded RNA interference of endogenous plant MMR.

106. A vector for introducing a dominant negative MMR allele into a plant, comprising: a dominant negative MMR allele under the transcriptional control of a promoter which is functional in a plant.

107. The vector of claim 106 wherein said vector further comprises an *Agrobacterium tumafaciens* T-DNA border repeat flanking the MMR allele.

108. The vector of claim 106 further comprising an origin of replication for independent replication in said plant.

109. The vector of claim 106 wherein the promoter is a Cauliflower Mosaic Virus promoter.

110. The vector of claim 106 wherein the promoter is a nopaline synthase promoter from *Agrobacterium tumafaciens*.

111. The vector of claim 106 further comprising a selectable marker.

112. The vector of claim 111 wherein the selectable marker is a neomycin phosphotransferase gene.

113. The vector of claim 106 wherein the MMR allele is PMS134.

114. The vector of claim 106 wherein the MMR allele is human PMS134.

115. The vector of claim 106 wherein the MMR allele is Arabidopsis PMS134.

116. An isolated and purified polynucleotide encoding Arabidopsis PMS2 as shown in SEQ ID NO: 14.

117. The isolated and purified polynucleotide of claim 116 comprising the sequence as shown in SEQ ID NO: 4.

118. An isolated and purified polynucleotide encoding Arabidopsis PMS134 as shown in SEQ ID NO: 16.

119. The isolated and purified polynucleotide of claim 118 comprising the sequence as shown in SEQ ID NO: 6.

120. An isolated and purified protein which is Arabidopsis PMS2 as shown in SEQ ID NO: 14.

121. An isolated and purified protein which is Arabidopsis PMS134 as shown in SEQ ID NO: 16.

122. A method for determining the presence of a mismatch repair (MMR) defect in a plant or a plant cell, comprising:

comparing at least two microsatellite markers in test cells or a test plant to the at least two microsatellite markers in cells of a normal plant;

identifying the test cells or test plant as having a mismatch repair defect if at least two microsatellite markers are found to be rearranged relative to the cells of the normal plant.

123. The method of claim 122 wherein a test plant is identified if at least one quarter of the markers compared are found to be rearranged.

124. The method of claim 122 wherein a test plant is identified if at least one third of the markers compared are found to be rearranged.

125. The method of claim 122 wherein a test plant is identified if at least one half of the markers compared are found to be rearranged.

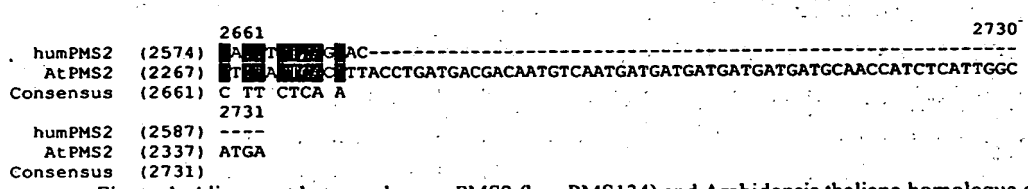



Figure 1. Alignment between human PMS2 (humPMS134) and Arabidopsis thaliana homologue of PMS2 (AtPMS2) DNA sequences. Similarity is 48.1%; identity is 48.1%. Black boxes show identical nucleotides.

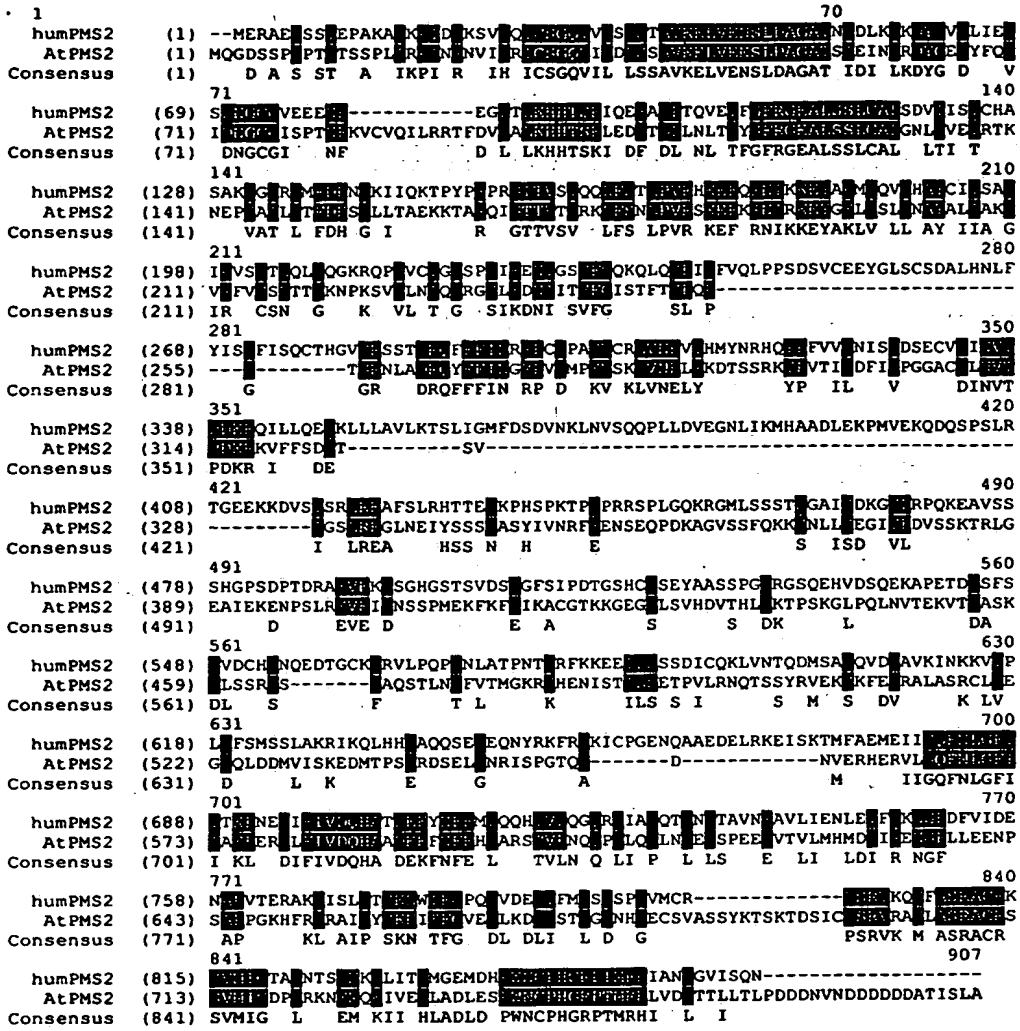
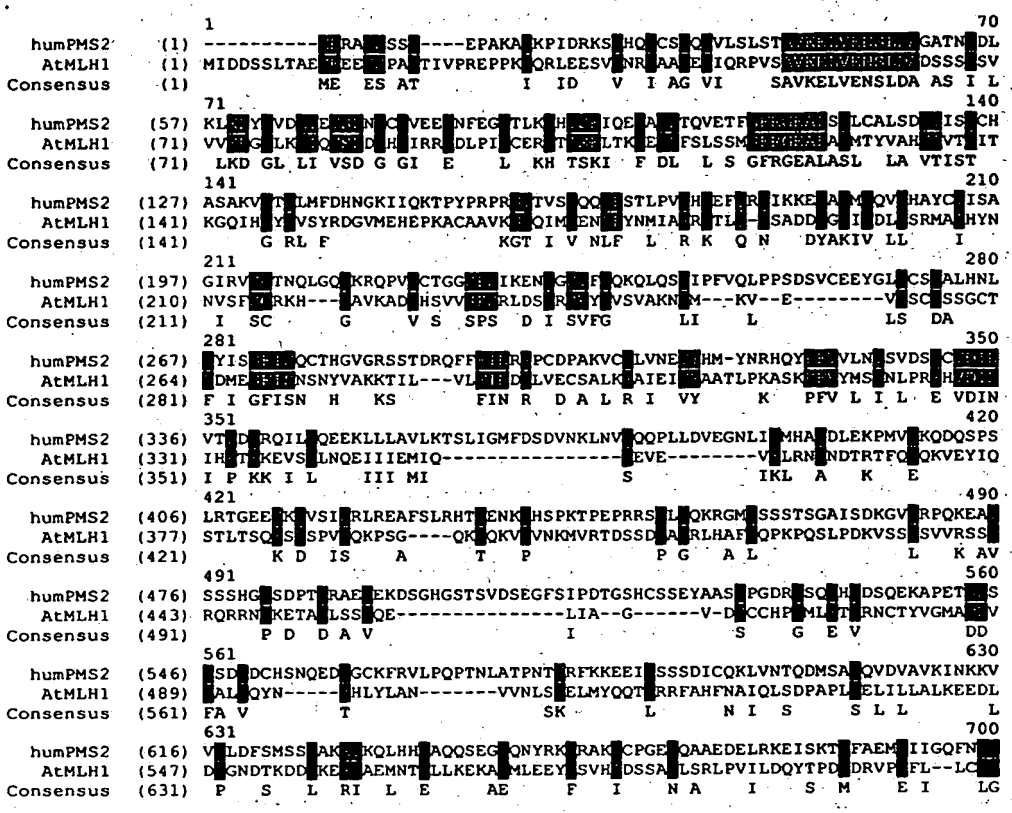


Figure 2. Alignment between human PMS2 (humPMS134) and Arabidopsis thaliana homologue of PMS2 (AtPMS2) amino acid sequences. Similarity is 41.5%; identity is 31.1%. Black boxes show identical residues.



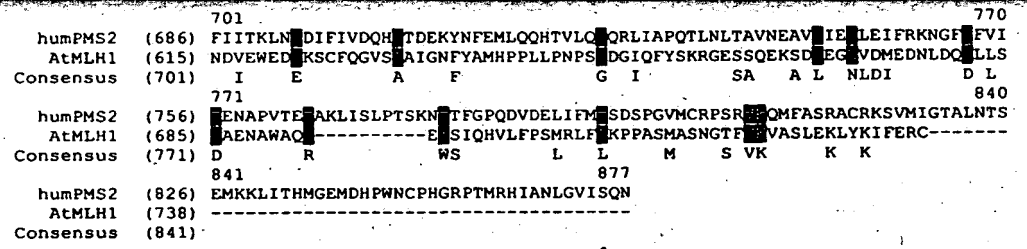


Figure 3. Alignment between human PMS2 (humPMS2) and Arabidopsis thaliana PMS2 homologue MLH1 (AtMLH1) amino acid sequences. Similarity is 30%; identity is 18.4%. Black boxes show identical residues.

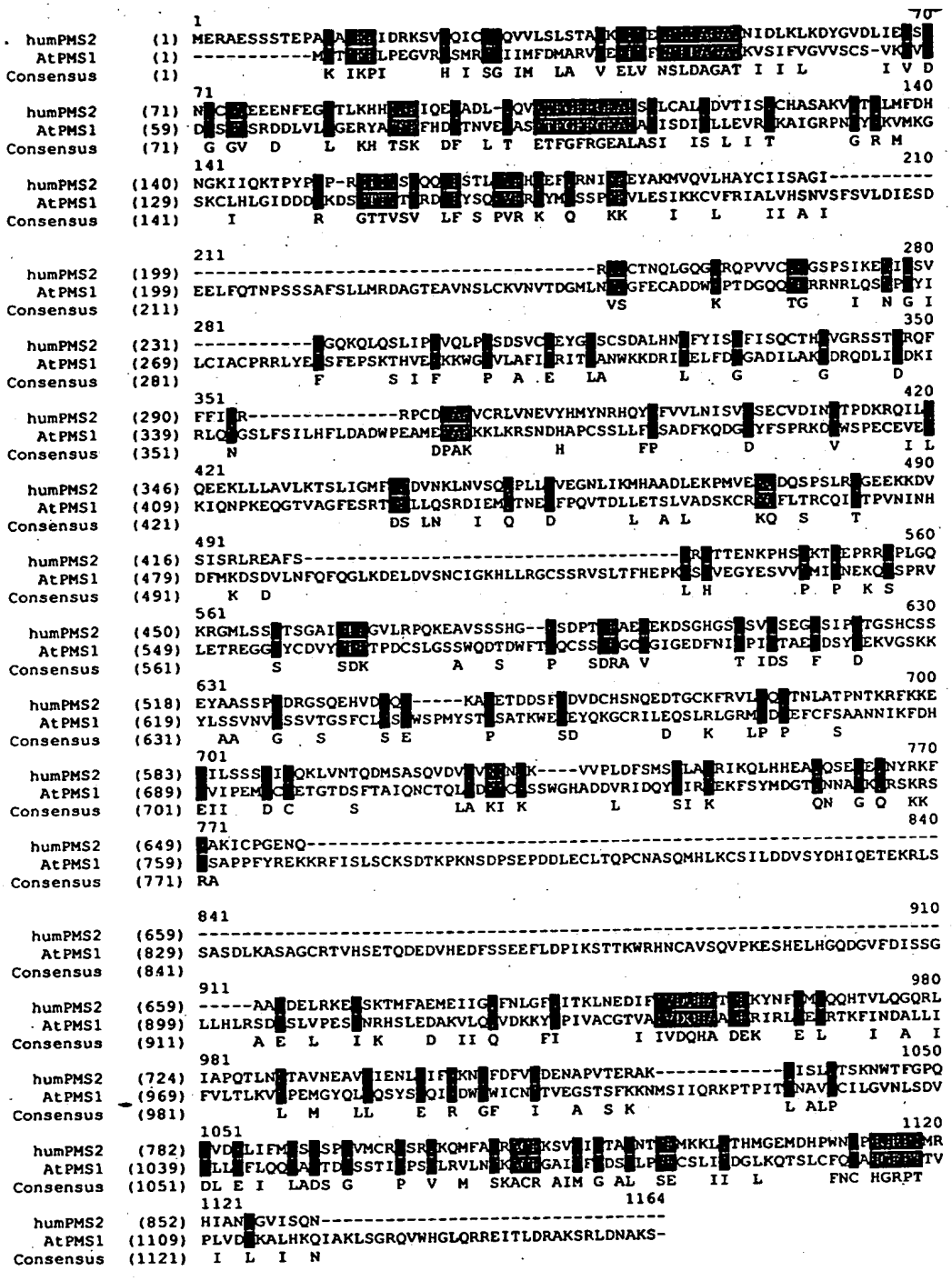


Figure 4. Alignment between human PMS2 (humPMS2) and Arabidopsis thaliana PMS2 homologue PMS1 (AtPMS1) amino acid sequences. Similarity is 24.4%; identity is 15%. Black boxes show identical residues.

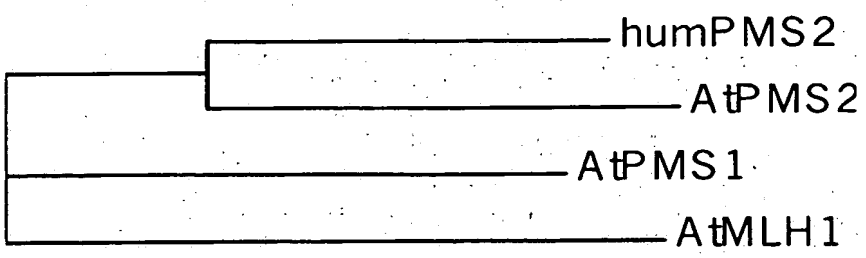



**Figure 5.** Phylogenetic tree of the *Arabidopsis thaliana PMS2* gene homologues.

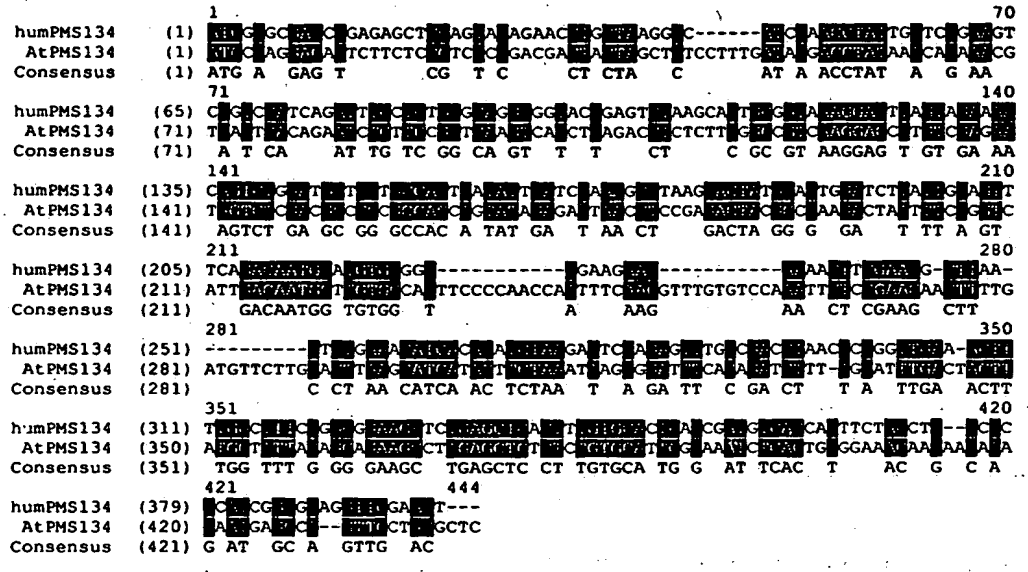



**Figure 6.** Alignment between human PMS134 (humPMS134) and Arabidopsis thaliana homologue of PMS134 (AtPMS134) DNA sequences. Similarity is 53.2%; identity is 53.2%. Black boxes show identical nucleotides.

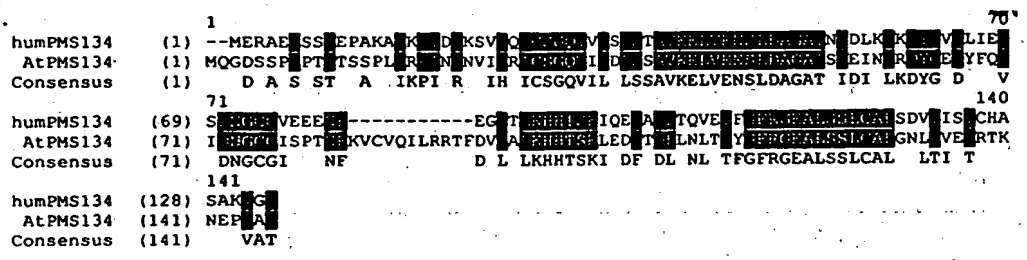



**Figure 7.** Alignment between human PMS134 (humPMS134) and Arabidopsis thaliana homologue of PMS134 (AtPMS134) amino acid sequences. Similarity is 65.1%; identity is 50.7%. Black boxes show identical residues.

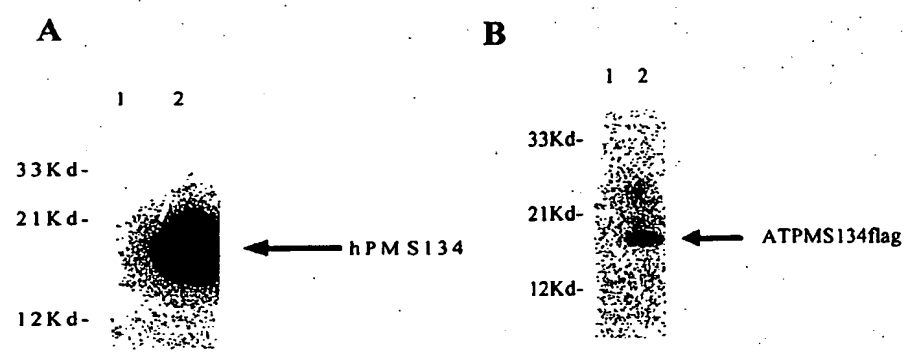



**Figure 8:** Western blot analysis of bacteria expressing the human PMS134 dominant negative gene (Panel A, lane 2) or the Arabidopsis thaliana dominant negative gene (Panel B, lane 2). Panel A, lysates from bacteria were loaded onto SDS-PAGE gels and probed with an antibody against the human PMS2 N-terminus. Panel B, lysates from bacteria were loaded onto SDS-PAGE gels and probed with an antibody against the flag epitope placed on the C-terminus of the Arabidopsis PMS134 gene. Lane 1 is bacteria containing empty vector as negative control.

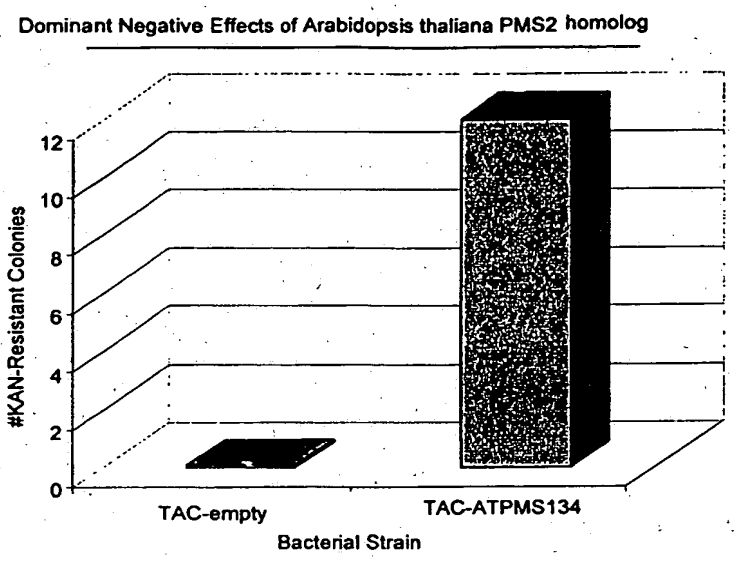



**Figure 9.** Expression of the *Arabidopsis thaliana PMS134* gene produces hypermutability in bacteria leading to the generation of new phenotypes. Briefly, bacteria containing the empty vector or the TAC ATPMS134 expression vector were grown and plated on kanamycin containing Lbagar plates. The host bacteria are susceptible to KAN bactericidal activity. Bacterial cultures expressing the hPMS134 gene resulted in genetic alteration of the bacterial host and the generation of clones that are KAN resistant.

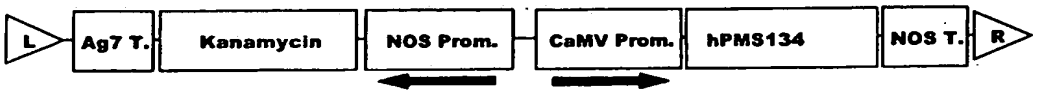



**Figure 10.** Schematic map of the pCMV-hPMS134-Kan binary plant expression vector. Ag7 T. and NOS T. = gene 7 and Nopaline Synthase poly(A) signals, respectively. NOS Prom and CaMV Prom = Nopaline Synthase and Cauliflower Mosaic Virus promoters, respectively. L and R = left and right T-DNA border repeats, respectively. Arrows indicate direction of transcription.

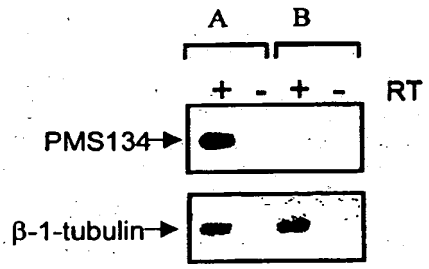



**Figure 11.** Expression of hPMS134 in Arabidopsis Thailana. Message analysis for T1 plants shows steady state expression of dominant negative MMR genes in PMS134-Kan plants (A) while none is observed in control plants (B). Tubulin was used as an internal control to monitor sample loading and integrity.

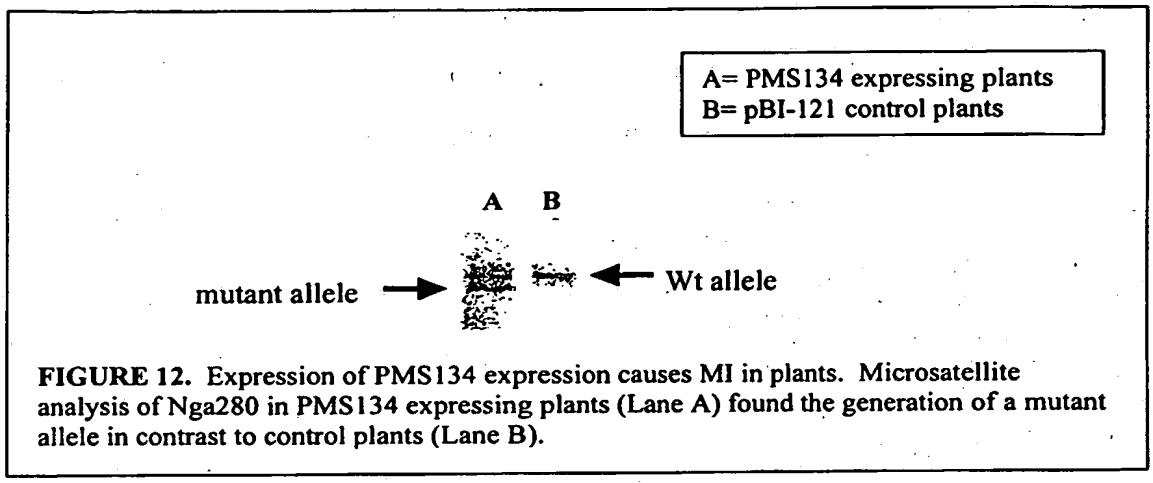
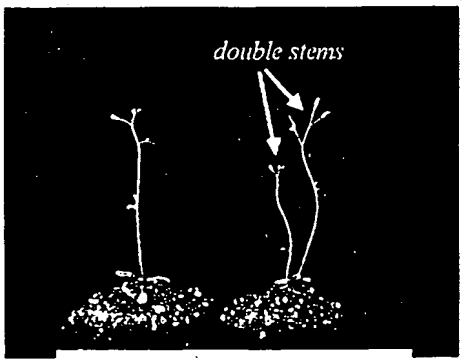



**FIGURE 12.** Expression of PMS134 expression causes MI in plants. Microsatellite analysis of Nga280 in PMS134 expressing plants (Lane A) found the generation of a mutant allele in contrast to control plants (Lane B).

**Figure 13.** The plant on the left is a wild type A. thaliana and the one on the right is MMR defective. Seeds from the MMR defective plant have been obtained and offspring have the same "double-meristem" trait.




Normal MMR-deficient

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<211> 737
<212> PRT
<213> Arabidopsis thaliana

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1 5 10 15

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Glu Glu Ser Val Val Asn Arg Ile Ala Ala Gly Glu Val Ile Gln Arg
        35                  40                  45
Pro Val Ser Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp Ala Asp
    50                  55                  60
Ser Ser Ser Ile Ser Val Val Val Lys Asp Gly Gly Leu Lys Leu Ile
65                  70                  75                  80
Gln Val Ser Asp Asp Gly His Gly Ile Arg Arg Glu Asp Leu Pro Ile
                85                  90                  95
Leu Cys Glu Arg His Thr Thr Ser Lys Leu Thr Lys Phe Glu Asp Leu
            100                 105                 110
Phe Ser Leu Ser Ser Met Gly Phe Arg Gly Glu Ala Leu Ala Ser Met
        115                 120                 125
Thr Tyr Val Ala His Val Thr Val Thr Thr Ile Thr Lys Gly Gln Ile
    130                 135                 140
His Gly Tyr Arg Val Ser Tyr Arg Asp Gly Val Met Glu His Glu Pro
145                 150                 155                 160
Lys Ala Cys Ala Ala Val Lys Gly Thr Gln Ile Met Val Glu Asn Leu
                165                 170                 175
Phe Tyr Asn Met Ile Ala Arg Arg Lys Thr Leu Gln Asn Ser Ala Asp
            180                 185                 190
Asp Tyr Gly Lys Ile Val Asp Leu Leu Ser Arg Met Ala Ile His Tyr
        195                 200                 205
Asn Asn Val Ser Phe Ser Cys Arg Lys His Gly Ala Val Lys Ala Asp
    210                 215                 220
Val His Ser Val Val Ser Pro Ser Arg Leu Asp Ser Ile Arg Ser Val
225                 230                 235                 240
Tyr Gly Val Ser Val Ala Lys Asn Leu Met Lys Val Glu Val Ser Ser
                245                 250                 255
Cys Asp Ser Ser Gly Cys Thr Phe Asp Met Glu Gly Phe Ile Ser Asn
            260                 265                 270
Ser Asn Tyr Val Ala Lys Lys Thr Ile Leu Val Leu Phe Ile Asn Asp
        275                 280                 285
Arg Leu Val Glu Cys Ser Ala Leu Lys Arg Ala Ile Glu Ile Val Tyr
    290                 295                 300
Ala Ala Thr Leu Pro Lys Ala Ser Lys Pro Phe Val Tyr Met Ser Ile
305                 310                 315                 320
Asn Leu Pro Arg Glu His Val Asp Ile Asn Ile His Pro Thr Lys Lys
                325                 330                 335
Glu Val Ser Leu Leu Asn Gln Glu Ile Ile Ile Glu Met Ile Gln Ser
            340                 345                 350
Glu Val Glu Val Lys Leu Arg Asn Ala Asn Asp Thr Arg Thr Phe Gln
        355                 360                 365
Glu Gln Lys Val Glu Tyr Ile Gln Ser Thr Leu Thr Ser Gln Lys Ser
    370                 375                 380
Asp Ser Pro Val Ser Gln Lys Pro Ser Gly Gln Lys Thr Gln Lys Val
385                 390                 395                 400
Pro Val Asn Lys Met Val Arg Thr Asp Ser Ser Asp Pro Ala Gly Arg
                405                 410                 415
Leu His Ala Phe Leu Gln Pro Lys Pro Gln Ser Leu Pro Asp Lys Val
            420                 425                 430
Ser Ser Leu Ser Val Val Arg Ser Ser Val Arg Gln Arg Arg Asn Pro
        435                 440                 445
Lys Glu Thr Ala Asp Leu Ser Ser Val Gln Glu Leu Ile Ala Gly Val
    450                 455                 460
Asp Ser Cys Cys His Pro Gly Met Leu Glu Thr Val Arg Asn Cys Thr
465                 470                 475                 480
Tyr Val Gly Met Ala Asp Asp Val Phe Ala Leu Val Gln Tyr Asn Thr
                485                 490                 495
His Leu Tyr Leu Ala Asn Val Val Asn Leu Ser Lys Glu Leu Met Tyr
            500                 505                 510
Gln Gln Thr Leu Arg Arg Phe Ala His Phe Asn Ala Ile Gln Leu Ser
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        515                 520                 525
Asp Pro Ala Pro Leu Ser Glu Leu Ile Leu Leu Ala Leu Lys Glu Glu
    530                 535                 540
Asp Leu Asp Pro Gly Asn Asp Thr Lys Asp Asp Leu Lys Glu Arg Ile
545                 550                 555                 560
Ala Glu Met Asn Thr Glu Leu Leu Lys Glu Lys Ala Glu Met Leu Glu
                565                 570                 575
Glu Tyr Phe Ser Val His Ile Asp Ser Ser Ala Asn Leu Ser Arg Leu
            580                 585                 590
Pro Val Ile Leu Asp Gln Tyr Thr Pro Asp Met Asp Arg Val Pro Glu
        595                 600                 605
Phe Leu Leu Cys Leu Gly Asn Asp Val Glu Trp Glu Asp Glu Lys Ser
    610                 615                 620
Cys Phe Gln Gly Val Ser Ala Ala Ile Gly Asn Phe Tyr Ala Met His
625                 630                 635                 640
Pro Pro Leu Leu Pro Asn Pro Ser Gly Asp Gly Ile Gln Phe Tyr Ser
                645                 650                 655
Lys Arg Gly Glu Ser Ser Gln Glu Lys Ser Asp Leu Glu Gly Asn Val
            660                 665                 670
Asp Met Glu Asp Asn Leu Asp Gln Asp Leu Leu Ser Asp Ala Glu Asn
        675                 680                 685
Ala Trp Ala Gln Arg Glu Trp Ser Ile Gln His Val Leu Phe Pro Ser
    690                 695                 700
Met Arg Leu Phe Leu Lys Pro Pro Ala Ser Met Ala Ser Asn Gly Thr
705                 710                 715                 720
Phe Val Lys Val Ala Ser Leu Glu Lys Leu Tyr Lys Ile Phe Glu Arg
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Cys
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<212> PRT
<213> Arabidopsis thaliana

<400> 10

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            20                  25                  30
Phe Asn Ser Leu Asp Ala Gly Ala Thr Lys Val Ser Ile Phe Val Gly
        35                  40                  45
Val Val Ser Cys Ser Val Lys Val Val Asp Asp Gly Ser Gly Val Ser
    50                  55                  60
Arg Asp Asp Leu Val Leu Leu Gly Glu Arg Tyr Ala Thr Ser Lys Phe
65                  70                  75                  80
His Asp Phe Thr Asn Val Glu Thr Ala Ser Glu Thr Phe Gly Phe Arg
                85                  90                  95
Gly Glu Ala Leu Ala Ser Ile Ser Asp Ile Ser Leu Leu Glu Val Arg
            100                 105                 110
Thr Lys Ala Ile Gly Arg Pro Asn Gly Tyr Arg Lys Val Met Lys Gly
        115                 120                 125
Ser Lys Cys Leu His Leu Gly Ile Asp Asp Asp Arg Lys Asp Ser Gly
    130                 135                 140
Thr Thr Val Thr Val Arg Asp Leu Phe Tyr Ser Gln Pro Val Arg Arg
145                 150                 155                 160
Lys Tyr Met Gln Ser Ser Pro Lys Lys Val Leu Glu Ser Ile Lys Lys
                165                 170                 175
Cys Val Phe Arg Ile Ala Leu Val His Ser Asn Val Ser Phe Ser Val
            180                 185                 190
Leu Asp Ile Glu Ser Asp Glu Glu Leu Phe Gln Thr Asn Pro Ser Ser
        195                 200                 205
Ser Ala Phe Ser Leu Leu Met Arg Asp Ala Gly Thr Glu Ala Val Asn
```

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    210                 215                 220
Ser Leu Cys Lys Val Asn Val Thr Asp Gly Met Leu Asn Val Ser Gly
225                 230                 235                 240
Phe Glu Cys Ala Asp Asp Trp Lys Pro Thr Asp Gly Gln Gln Thr Gly
                245                 250                 255
Arg Arg Asn Arg Leu Gln Ser Asn Pro Gly Tyr Ile Leu Cys Ile Ala
            260                 265                 270
Cys Pro Arg Arg Leu Tyr Glu Phe Ser Phe Glu Pro Ser Lys Thr His
        275                 280                 285
Val Glu Phe Lys Lys Trp Gly Pro Val Leu Ala Phe Ile Glu Arg Ile
    290                 295                 300
Thr Leu Ala Asn Trp Lys Lys Asp Arg Ile Leu Glu Leu Phe Asp Gly
305                 310                 315                 320
Gly Ala Asp Ile Leu Ala Lys Gly Asp Arg Gln Asp Leu Ile Asp Asp
                325                 330                 335
Lys Ile Arg Leu Gln Asn Gly Ser Leu Phe Ser Ile Leu His Phe Leu
            340                 345                 350
Asp Ala Asp Trp Pro Glu Ala Met Glu Pro Ala Lys Lys Lys Leu Lys
        355                 360                 365
Arg Ser Asn Asp His Ala Pro Cys Ser Ser Leu Leu Phe Pro Ser Ala
    370                 375                 380
Asp Phe Lys Gln Asp Gly Asp Tyr Phe Ser Pro Arg Lys Asp Val Trp
385                 390                 395                 400
Ser Pro Glu Cys Glu Val Glu Leu Lys Ile Gln Asn Pro Lys Glu Gln
                405                 410                 415
Gly Thr Val Ala Gly Phe Glu Ser Arg Thr Asp Ser Leu Leu Gln Ser
            420                 425                 430
Arg Asp Ile Glu Met Gln Thr Asn Glu Asp Phe Pro Gln Val Thr Asp
        435                 440                 445
Leu Leu Glu Thr Ser Leu Val Ala Asp Ser Lys Cys Arg Lys Gln Phe
    450                 455                 460
Leu Thr Arg Cys Gln Ile Thr Thr Pro Val Asn Ile Asn His Asp Phe
465                 470                 475                 480
Met Lys Asp Ser Asp Val Leu Asn Phe Gln Phe Gln Gly Leu Lys Asp
                485                 490                 495
Glu Leu Asp Val Ser Asn Cys Ile Gly Lys His Leu Leu Arg Gly Cys
            500                 505                 510
Ser Ser Arg Val Ser Leu Thr Phe His Glu Pro Lys Leu Ser His Val
        515                 520                 525
Glu Gly Tyr Glu Ser Val Val Pro Met Ile Pro Asn Glu Lys Gln Ser
    530                 535                 540
Ser Pro Arg Val Leu Glu Thr Arg Glu Gly Gly Ser Tyr Cys Asp Val
545                 550                 555                 560
Tyr Ser Asp Lys Thr Pro Asp Cys Ser Leu Gly Ser Ser Trp Gln Asp
                565                 570                 575
Thr Asp Trp Phe Thr Pro Gln Cys Ser Ser Asp Arg Gly Cys Val Gly
            580                 585                 590
Ile Gly Glu Asp Phe Asn Ile Thr Pro Ile Asp Thr Ala Glu Phe Asp
        595                 600                 605
Ser Tyr Asp Glu Lys Val Gly Ser Lys Lys Tyr Leu Ser Ser Val Asn
    610                 615                 620
Val Gly Ser Ser Val Thr Gly Ser Phe Cys Leu Ser Ser Glu Trp Ser
625                 630                 635                 640
Pro Met Tyr Ser Thr Pro Ser Ala Thr Lys Trp Glu Ser Glu Tyr Gln
                645                 650                 655
Lys Gly Cys Arg Ile Leu Glu Gln Ser Leu Arg Leu Gly Arg Met Pro
            660                 665                 670
Asp Pro Glu Phe Cys Phe Ser Ala Ala Asn Asn Ile Lys Phe Asp His
        675                 680                 685
Glu Val Ile Pro Glu Met Asp Cys Cys Glu Thr Gly Thr Asp Ser Phe
    690                 695                 700
Thr Ala Ile Gln Asn Cys Thr Gln Leu Ala Asp Lys Ile Cys Lys Ser
705                 710                 715                 720
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Ser Trp Gly His Ala Asp Asp Val Arg Ile Asp Gln Tyr Ser Ile Arg
                725                 730                 735
Lys Glu Lys Phe Ser Tyr Met Asp Gly Thr Gln Asn Asn Ala Gly Lys
            740                 745                 750
Gln Arg Ser Lys Arg Ser Arg Ser Ala Pro Pro Phe Tyr Arg Glu Lys
        755                 760                 765
Lys Arg Phe Ile Ser Leu Ser Cys Lys Ser Asp Thr Lys Pro Lys Asn
    770                 775                 780
Ser Asp Pro Ser Glu Pro Asp Asp Leu Glu Cys Leu Thr Gln Pro Cys
785                 790                 795                 800
Asn Ala Ser Gln Met His Leu Lys Cys Ser Ile Leu Asp Asp Val Ser
                805                 810                 815
Tyr Asp His Ile Gln Glu Thr Glu Lys Arg Leu Ser Ser Ala Ser Asp
            820                 825                 830
Leu Lys Ala Ser Ala Gly Cys Arg Thr Val His Ser Glu Thr Gln Asp
        835                 840                 845
Glu Asp Val His Glu Asp Phe Ser Ser Glu Glu Phe Leu Asp Pro Ile
    850                 855                 860
Lys Ser Thr Thr Lys Trp Arg His Asn Cys Ala Val Ser Gln Val Pro
865                 870                 875                 880
Lys Glu Ser His Glu Leu His Gly Gln Asp Gly Val Phe Asp Ile Ser
                885                 890                 895
Ser Gly Leu Leu His Leu Arg Ser Asp Glu Ser Leu Val Pro Glu Ser
            900                 905                 910
Ile Asn Arg His Ser Leu Glu Asp Ala Lys Val Leu Gln Gln Val Asp
        915                 920                 925
Lys Lys Tyr Ile Pro Ile Val Ala Cys Gly Thr Val Ala Ile Val Asp
    930                 935                 940
Gln His Ala Ala Asp Glu Arg Ile Arg Leu Glu Glu Leu Arg Thr Lys
945                 950                 955                 960
Phe Ile Asn Asp Ala Leu Leu Ile Phe Val Leu Thr Leu Lys Val Leu
                965                 970                 975
Pro Glu Met Gly Tyr Gln Leu Leu Gln Ser Tyr Ser Glu Gln Ile Arg
            980                 985                 990
Asp Trp Gly Trp Ile Cys Asn Ile Thr Val Glu Gly Ser Thr Ser Phe
        995                 1000                1005
Lys Lys Asn Met Ser Ile Ile Gln Arg Lys Pro Thr Pro Ile Thr Leu
    1010                1015                1020
Asn Ala Val Pro Cys Ile Leu Gly Val Asn Leu Ser Asp Val Asp Leu
1025                1030                1035                1040
Leu Glu Phe Leu Gln Gln Leu Ala Asp Thr Asp Gly Ser Ser Thr Ile
                1045                1050                1055
Pro Pro Ser Val Leu Arg Val Leu Asn Ser Lys Ala Cys Arg Gly Ala
            1060                1065                1070
Ile Met Phe Gly Asp Ser Leu Leu Pro Ser Glu Cys Ser Leu Ile Ile
        1075                1080                1085
Asp Gly Leu Lys Gln Thr Ser Leu Cys Phe Gln Cys Ala His Gly Arg
    1090                1095                1100
Pro Thr Thr Val Pro Leu Val Asp Leu Lys Ala Leu His Lys Gln Ile
1105                1110                1115                1120
Ala Lys Leu Ser Gly Arg Gln Val Trp His Gly Leu Gln Arg Arg Glu
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            1140                1145                1150
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<211> 862
<212> PRT
<213> Homo sapiens

<400> 11

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Leu Ser Leu Ser Thr Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp
        35                  40                  45
Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
    50                  55                  60
Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Glu Asn Phe
65                  70                  75                  80
Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
                85                  90                  95
Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
            100                 105                 110
Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
        115                 120                 125
Ala Lys Val Gly Thr Arg Leu Met Phe Asp His Asn Gly Lys Ile Ile
    130                 135                 140
Gln Lys Thr Pro Tyr Pro Arg Pro Arg Gly Thr Thr Val Ser Val Gln
145                 150                 155                 160
Gln Leu Phe Ser Thr Leu Pro Val Arg His Lys Glu Phe Gln Arg Asn
                165                 170                 175
Ile Lys Lys Glu Tyr Ala Lys Met Val Gln Val Leu His Ala Tyr Cys
            180                 185                 190
Ile Ile Ser Ala Gly Ile Arg Val Ser Cys Thr Asn Gln Leu Gly Gln
        195                 200                 205
Gly Lys Arg Gln Pro Val Val Cys Thr Gly Gly Ser Pro Ser Ile Lys
    210                 215                 220
Glu Asn Ile Gly Ser Val Phe Gly Gln Lys Gln Leu Gln Ser Leu Ile
225                 230                 235                 240
Pro Phe Val Gln Leu Pro Pro Ser Asp Ser Val Cys Glu Glu Tyr Gly
                245                 250                 255
Leu Ser Cys Ser Asp Ala Leu His Asn Leu Phe Tyr Ile Ser Gly Phe
            260                 265                 270
Ile Ser Gln Cys Thr His Gly Val Gly Arg Ser Ser Thr Asp Arg Gln
        275                 280                 285
Phe Phe Phe Ile Asn Arg Arg Pro Cys Asp Pro Ala Lys Val Cys Arg
    290                 295                 300
Leu Val Asn Glu Val Tyr His Met Tyr Asn Arg His Gln Tyr Pro Phe
305                 310                 315                 320
Val Val Leu Asn Ile Ser Val Asp Ser Glu Cys Val Asp Ile Asn Val
                325                 330                 335
Thr Pro Asp Lys Arg Gln Ile Leu Leu Gln Glu Glu Lys Leu Leu Leu
            340                 345                 350
Ala Val Leu Lys Thr Ser Leu Ile Gly Met Phe Asp Ser Asp Val Asn
        355                 360                 365
Lys Leu Asn Val Ser Gln Gln Pro Leu Leu Asp Val Glu Gly Asn Leu
    370                 375                 380
Ile Lys Met His Ala Ala Asp Leu Glu Lys Pro Met Val Glu Lys Gln
385                 390                 395                 400
Asp Gln Ser Pro Ser Leu Arg Thr Gly Glu Glu Lys Lys Asp Val Ser
                405                 410                 415
Ile Ser Arg Leu Arg Glu Ala Phe Ser Leu Arg His Thr Thr Glu Asn
            420                 425                 430
Lys Pro His Ser Pro Lys Thr Pro Glu Pro Arg Arg Ser Pro Leu Gly
        435                 440                 445
Gln Lys Arg Gly Met Leu Ser Ser Ser Thr Ser Gly Ala Ile Ser Asp
    450                 455                 460
Lys Gly Val Leu Arg Pro Gln Lys Glu Ala Val Ser Ser Ser His Gly
465                 470                 475                 480
Pro Ser Asp Pro Thr Asp Arg Ala Glu Val Glu Lys Asp Ser Gly His
                485                 490                 495
Gly Ser Thr Ser Val Asp Ser Glu Gly Phe Ser Ile Pro Asp Thr Gly
            500                 505                 510
Ser His Cys Ser Ser Glu Tyr Ala Ala Ser Ser Pro Gly Asp Arg Gly
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        515                 520                 525
Ser Gln Glu His Val Asp Ser Gln Glu Lys Ala Pro Glu Thr Asp Asp
    530                 535                 540
Ser Phe Ser Asp Val Asp Cys His Ser Asn Gln Glu Asp Thr Gly Cys
545                 550                 555                 560
Lys Phe Arg Val Leu Pro Gln Pro Thr Asn Leu Ala Thr Pro Asn Thr
                565                 570                 575
Lys Arg Phe Lys Lys Glu Glu Ile Leu Ser Ser Ser Asp Ile Cys Gln
            580                 585                 590
Lys Leu Val Asn Thr Gln Asp Met Ser Ala Ser Gln Val Asp Val Ala
        595                 600                 605
Val Lys Ile Asn Lys Lys Val Val Pro Leu Asp Phe Ser Met Ser Ser
    610                 615                 620
Leu Ala Lys Arg Ile Lys Gln Leu His His Glu Ala Gln Gln Ser Glu
625                 630                 635                 640
Gly Glu Gln Asn Tyr Arg Lys Phe Arg Ala Lys Ile Cys Pro Gly Glu
                645                 650                 655
Asn Gln Ala Ala Glu Asp Glu Leu Arg Lys Glu Ile Ser Lys Thr Met
            660                 665                 670
Phe Ala Glu Met Glu Ile Ile Gly Gln Phe Asn Leu Gly Phe Ile Ile
        675                 680                 685
Thr Lys Leu Asn Glu Asp Ile Phe Ile Val Asp Gln His Ala Thr Asp
    690                 695                 700
Glu Lys Tyr Asn Phe Glu Met Leu Gln Gln His Thr Val Leu Gln Gly
705                 710                 715                 720
Gln Arg Leu Ile Ala Pro Gln Thr Leu Asn Leu Thr Ala Val Asn Glu
                725                 730                 735
Ala Val Leu Ile Glu Asn Leu Glu Ile Phe Arg Lys Asn Gly Phe Asp
            740                 745                 750
Phe Val Ile Asp Glu Asn Ala Pro Val Thr Glu Arg Ala Lys Leu Ile
        755                 760                 765
Ser Leu Pro Thr Ser Lys Asn Trp Thr Phe Gly Pro Gln Asp Val Asp
    770                 775                 780
Glu Leu Ile Phe Met Leu Ser Asp Ser Pro Gly Val Met Cys Arg Pro
785                 790                 795                 800
Ser Arg Val Lys Gln Met Phe Ala Ser Arg Ala Cys Arg Lys Ser Val
                805                 810                 815
Met Ile Gly Thr Ala Leu Asn Thr Ser Glu Met Lys Lys Leu Ile Thr
            820                 825                 830
His Met Gly Glu Met Asp His Pro Trp Asn Cys Pro His Gly Arg Pro
        835                 840                 845
Thr Met Arg His Ile Ala Asn Leu Gly Val Ile Ser Gln Asn
    850                 855                 860
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<213> Arabidopsis thaliana

<400> 12

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Val Ile Leu Asp Leu Ser Ser Ala Val Lys Glu Leu Val Glu Asn Ser
        35                  40                  45
Leu Asp Ala Gly Ala Thr Ser Ile Glu Ile Asn Leu Arg Asp Tyr Gly
    50                  55                  60
Glu Asp Tyr Phe Gln Val Ile Asp Asn Gly Cys Gly Ile Ser Pro Thr
65                  70                  75                  80
Asn Phe Lys Val Cys Val Gln Ile Leu Arg Arg Thr Phe Asp Val Leu
                85                  90                  95
Ala Leu Lys His His Thr Ser Lys Leu Glu Asp Phe Thr Asp Leu Leu
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            100                 105                 110
Asn Leu Thr Thr Tyr Gly Phe Arg Gly Glu Ala Leu Ser Ser Leu Cys
        115                 120                 125
Ala Leu Gly Asn Leu Thr Val Glu Thr Arg Thr Lys Asn Glu Pro Val
    130                 135                 140
Ala Thr Leu Leu Thr Phe Asp His Ser Gly Leu Leu Thr Ala Glu Lys
145                 150                 155                 160
Lys Thr Ala Arg Gln Ile Gly Thr Thr Val Thr Val Arg Lys Leu Phe
                165                 170                 175
Ser Asn Leu Pro Val Arg Ser Lys Glu Phe Lys Arg Asn Ile Arg Lys
            180                 185                 190
Glu Tyr Gly Lys Leu Val Ser Leu Leu Asn Ala Tyr Ala Leu Ile Ala
        195                 200                 205
Lys Gly Val Arg Phe Val Cys Ser Asn Thr Thr Gly Lys Asn Pro Lys
    210                 215                 220
Ser Val Val Leu Asn Thr Gln Gly Arg Gly Ser Leu Lys Asp Asn Ile
225                 230                 235                 240
Ile Thr Val Phe Gly Ile Ser Thr Phe Thr Ser Leu Gln Pro Gly Thr
                245                 250                 255
Gly Arg Asn Leu Ala Asp Arg Gln Tyr Phe Phe Ile Asn Gly Arg Pro
            260                 265                 270
Val Asp Met Pro Lys Val Ser Lys Leu Val Asn Glu Leu Tyr Lys Asp
        275                 280                 285
Thr Ser Ser Arg Lys Tyr Pro Val Thr Ile Leu Asp Phe Ile Val Pro
    290                 295                 300
Gly Gly Ala Cys Asp Leu Asn Val Thr Pro Asp Lys Arg Lys Val Phe
305                 310                 315                 320
Phe Ser Asp Glu Thr Ser Val Ile Gly Ser Leu Arg Glu Gly Leu Asn
                325                 330                 335
Glu Ile Tyr Ser Ser Ser Asn Ala Ser Tyr Ile Val Asn Arg Phe Glu
            340                 345                 350
Glu Asn Ser Glu Gln Pro Asp Lys Ala Gly Val Ser Ser Phe Gln Lys
        355                 360                 365
Lys Ser Asn Leu Leu Ser Glu Gly Ile Val Leu Asp Val Ser Ser Lys
    370                 375                 380
Thr Arg Leu Gly Glu Ala Ile Glu Lys Glu Asn Pro Ser Leu Arg Glu
385                 390                 395                 400
Val Glu Ile Asp Asn Ser Ser Pro Met Glu Lys Phe Lys Phe Glu Ile
                405                 410                 415
Lys Ala Cys Gly Thr Lys Lys Gly Glu Gly Ser Leu Ser Val His Asp
            420                 425                 430
Val Thr His Leu Asp Lys Thr Pro Ser Lys Gly Leu Pro Gln Leu Asn
        435                 440                 445
Val Thr Glu Lys Val Thr Asp Ala Ser Lys Asp Leu Ser Ser Arg Ser
    450                 455                 460
Ser Phe Ala Gln Ser Thr Leu Asn Thr Phe Val Thr Met Gly Lys Arg
465                 470                 475                 480
Lys His Glu Asn Ile Ser Thr Ile Leu Ser Glu Thr Pro Val Leu Arg
                485                 490                 495
Asn Gln Thr Ser Ser Tyr Arg Val Glu Lys Ser Lys Phe Glu Val Arg
            500                 505                 510
Ala Leu Ala Ser Arg Cys Leu Val Glu Gly Asp Gln Leu Asp Asp Met
        515                 520                 525
Val Ile Ser Lys Glu Asp Met Thr Pro Ser Glu Arg Asp Ser Glu Leu
    530                 535                 540
Gly Asn Arg Ile Ser Pro Gly Thr Gln Ala Asp Asn Val Glu Arg His
545                 550                 555                 560
Glu Arg Val Leu Gly Gln Phe Asn Leu Gly Phe Ile Ile Ala Lys Leu
                565                 570                 575
Glu Arg Asp Leu Phe Ile Val Asp Gln His Ala Ala Asp Glu Lys Phe
            580                 585                 590
Asn Phe Glu His Leu Ala Arg Ser Thr Val Leu Asn Gln Gln Pro Leu
        595                 600                 605
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Leu Gln Pro Leu Asn Leu Glu Leu Ser Pro Glu Glu Glu Val Thr Val
    610                 615                 620
Leu Met His Met Asp Ile Ile Arg Glu Asn Gly Phe Leu Leu Glu Glu
625                 630                 635                 640
Asn Pro Ser Ala Pro Pro Gly Lys His Phe Arg Leu Arg Ala Ile Pro
                645                 650                 655
Tyr Ser Lys Asn Ile Thr Phe Gly Val Glu Asp Leu Lys Asp Leu Ile
            660                 665                 670
Ser Thr Leu Gly Asp Asn His Gly Glu Cys Ser Val Ala Ser Ser Tyr
        675                 680                 685
Lys Thr Ser Lys Thr Asp Ser Ile Cys Pro Ser Arg Val Arg Ala Met
    690                 695                 700
Leu Ala Ser Arg Ala Cys Arg Ser Ser Val Met Ile Gly Asp Pro Leu
705                 710                 715                 720
Arg Lys Asn Glu Met Gln Lys Ile Val Glu His Leu Ala Asp Leu Glu
                725                 730                 735
Ser Pro Trp Asn Cys Pro His Gly Arg Pro Thr Met Arg His Leu Val
            740                 745                 750
Asp Leu Thr Thr Leu Leu Thr Leu Pro Asp Asp Asp Asn Val Asn Asp
        755                 760                 765
Asp Asp Asp Asp Asp Ala Thr Ile Ser Leu Ala
    770                 775
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<210> 13
<211> 133
<212> PRT
<213> Homo sapiens

<400> 13

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Pro Ile Asp Arg Lys Ser Val His Gln Ile Cys Ser Gly Gln Val Val
            20                  25                  30
Leu Ser Leu Ser Thr Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp
        35                  40                  45
Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
    50                  55                  60
Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Glu Asn Phe
65                  70                  75                  80
Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
                85                  90                  95
Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
            100                 105                 110
Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
        115                 120                 125
Ala Lys Val Gly Thr
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<210> 14
<211> 147
<212> PRT
<213> Arabidopsis thaliana

<400> 14

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Met Gln Gly Asp Ser Ser Pro Ser Pro Thr Thr Thr Ser Ser Pro Leu
 1               5                  10                  15
Ile Arg Pro Ile Asn Arg Asn Val Ile His Arg Ile Cys Ser Gly Gln
            20                  25                  30
Val Ile Leu Asp Leu Ser Ser Ala Val Lys Glu Leu Val Glu Asn Ser
        35                  40                  45
Leu Asp Ala Gly Ala Thr Ser Ile Glu Ile Asn Leu Arg Asp Tyr Gly
    50                  55                  60
Glu Asp Tyr Phe Gln Val Ile Asp Asn Gly Cys Gly Ile Ser Pro Thr
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```
65                  70                  75                  80
Asn Phe Lys Val Cys Val Gln Ile Leu Arg Arg Thr Phe Asp Val Leu
                85                  90                  95
Ala Leu Lys His His Thr Ser Lys Leu Glu Asp Phe Thr Asp Leu Leu
            100                 105                 110
Asn Leu Thr Thr Tyr Gly Phe Arg Gly Glu Ala Leu Ser Ser Leu Cys
        115                 120                 125
Ala Leu Gly Asn Leu Thr Val Glu Thr Arg Thr Lys Asn Glu Pro Val
    130                 135                 140
Ala Thr Leu
145
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# INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 00/35397

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/29 C12N15/82 C12N5/10 C12Q1/68 C07K14/415 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 99 19492 A (BETZNER ANDREAS STEFAN ;DOUTRIAUX MARIE PASCALE (FR); PEREZ PASCAL) 22 April 1999 (1999-04-22) | 86, 91-105 |
| Y | the whole document | 1-90, 106-114 |
| | --- | |
| Y | NICOLAIDES NICHOLAS C ET AL: "A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype." MOLECULAR AND CELLULAR BIOLOGY, vol. 18, no. 3, March 1998 (1998-03), pages 1635-1641, XP002165242 ISSN: 0270-7306 the whole document, esp. p.1641, first paragraph | 1-90, 106-114 |
| | --- | |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 April 2001 | 09.07.01 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Kania, T |

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## INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 00/35397

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 97 05268 A (SETRATECH ;WIND NIELS DE (NL); DEKKER VLAAR HELENA MARIA JOHA (NL)) 13 February 1997 (1997-02-13) see the whole document; esp. claim 5 | 87-90 |
| A | K M CULLIGAN AND J B HAYS: "DNA mismatch repair in plants" PLANT PHYSIOLOGY,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD,US, vol. 115, 1997, pages 833-839, XP002099372 ISSN: 0032-0889 cited in the application the whole document | 1-114 |
| A | LIPKIN STEVEN M ET AL: "MLH3: A DNA mismatch repair gene associated with mammalian microsatellite instability." NATURE GENETICS, vol. 24, no. 1, January 2000 (2000-01), pages 27-35, XP002165243 ISSN: 1061-4036 page 33, left-hand column, line 8 - line 18 | 1-86,89, 90, 106-112 |
| A | JEAN M ET AL: "Isolation and characterization of AtMLH1, a MutL homologue from Arabidopsis thaliana." MOLECULAR AND GENERAL GENETICS, vol. 262, no. 4-5, December 1999 (1999-12), pages 633-642, XP000986138 ISSN: 0026-8925 the whole document | 1-114 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US 00/35397

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-114

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-114

Methods for making hypermutable plants cells by introducing a polynucleotide comprising a dominant-negative allele of a mismatch repair gene into the cell, whereby the cell becomes hypermutable. Said method as embodied in the claims, and homogeneous compositions of hypermutable plant cells generated by said method.
A hypermutable transgenic plant wherein at least 50% of the cells comprise a dominant-negative allele of a mismatch repair gene, said gene as embodied in the claims.
A method of generating a mutation in a gene of interest in a plant cell or a plant comprising the steps of: growing a hypermutable plant cell or plant comprising the gene of interest and a dominant-negative allele of a mismatch repair gene, testing the plant cell or plant to determine whether the gene of interest harbors a mutation. Said step of testing as embodied in the claims. A hypermutable plant made by said method and as embodied in the claims.
A method for generating a hypermutable plant comprising the steps of: inhibiting endogenous mismatch repair activity of a plant, whereby the plant becomes hypermutable. Said step of inhibiting and the mismatch repair sequences used as embodied in the claims.
A vector for introducing a dominant-negative allele of a mismatch repair gene into a plant comprising: a dominant-negative allele of a mismatch repair gene under the transcriptional control of a promoter which is functional in a plant. Said vector as embodied in the claims.

2. Claims: 115-121

A vector for introducing a dominant-negative allele of a mismatch repair gene into a plant comprising: a dominant-negative allele of a mismatch repair gene under the transcriptional control of a promoter which is functional in a plant. Said vector wherein the allele of the mismatch repair gene is Arabidopsis PMS134.
An isolated and purified polynucleotide encoding Arabidopsis PMS2 as shown in SEQ ID NO:14, and comprising the sequence as shown in SEQ ID NO:4.
An isolated and purified polynucleotide encoding Arabidopsis PMS134 as shown in SEQ ID NO:16, and comprising the sequence as shown in SEQ ID NO:6.
Isolated and purified proteins which are Arabidopsis PMS2 and PMS134 as shown in SEQ ID NOs:14,16.

3. Claim : 122

A method for determining the presence of a mismatch repair

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

defect in a plant or a plant cell, comprising: comparing at least two microsatellite markers in test cells or a test plant to the at least two markers in cells of a normal plant, identifying the test cells or test plant as having a mismatch repair defect if at least two markers are found to be rearranged relative to the cells of a normal plant. Said method as embodied in the claims.

# INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter ial Application No
PC I7US 00/35397

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9919492 A | 22-04-1999 | AU 1157399 A | 03-05-1999 |
| | | EP 1027447 A | 16-08-2000 |
| | | ZA 9809242 A | 15-04-1999 |
| WO 9705268 A | 13-02-1997 | AU 4784996 A | 26-02-1997 |
| | | EP 0842289 A | 20-05-1998 |